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1977
pt.2-A

NATIONAL CANCER INSTITUTE

ANNUAL REPORT FY 1977

July 1, 1976 through September 30, 1977

Part II-A

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

Report of program activities

U S NATIONAL CANCER INSTITUTE

ANNUAL REPORT

July 1, 1976 through September 30, 1977

TABLE OF CONTENTS

		Page
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS		
DIRECTOR		
Summary Report		XXXVIII
Laboratory of Biochemistry		
Summary Report		1
Project Reports:		
CB-5210	Cellular Controls over Growth and Inducible Processes	16
CB-366	Biosynthesis and Assembly of Intracellular Components	23
CB-379	Ultrastructural Characterization of Macromolecules	26
CB-945	Study of the Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells	29
CB-5214	DNA Synthesis in Mammalian Cells	35
CB-5215	Biological and Ultrastructural Studies on Cell Cooperation in the Immune Response	39
CB-5241	Transcription of Covalently Linked Cellular and SV40 Viral DNA	43
CB-3212	Studies on Cell Nutrition, Growth and Neoplastic Transformation In Vitro	45
CB-3213	Physiology and Carcinogenesis of Mammalian Cells in Culture	49

RC
267
U5626
1977
Pt. 2-A

CB-3234	Primary Biochemical Alterations Leading to Neoplastic Conversion	54
CB-3257	Growth and Neoplastic Transformation of Mammalian Cells In Vitro	57
CB-3264	Spontaneous Neoplastic Change During Growth in Culture	61
CB-5228	The Cytology of Neoplastic Transformation In Vitro	62
CB-5239	Carcinomatous Change in Human Cells Studied In Vitro	65
CB-5238	Effect of Estrogen on Gene Expression in the Avian Liver	68
CB-5240	The Synthesis of Vitellogenin by Avian Liver Cells in Culture	72
CB-5242	Organization of Estrogen-Responsive Genes in The Avian Genome	74
CB-5243	In Vitro Synthesis of Vitellogenin Messenger RNA	78
CB-5231	Role of Subunit Interactions in Enzyme Chemistry	82
CB-5232	Studies on the Replication of Simian Virus 40 (SV40) DNA in Monkey Kidney Cells	86
CB-5234	Replication and Properties Defective - Substituted Simian Virus 40 in Permissive Moneky Kidney Cells	89
CB-5237	Interaction of Histones with Superhelical DNA	94
CB-333	The Biochemical Basis for Defective Muturation in Myelogenous Leukemia	99
CB-375	Homogeneity and Structure of Proteins	103
CB-5202	Isolation, Fractionation, and Characterization of Native Nucleoproteins	107
CB-5203	Immunocnemical and Physical Purifications of Immunocytes and Their Components	111

Laboratory of Molecular Biology

Summary Report		115
Project Reports:		
CB-8000	Regulation of Gene Activity by cAMP and cGMP	119
CB-8001	Role of Cyclic AMP in the Regulation of Growth and Morphology of Transformed Cells	122
CB-8712	The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior	127
CB-8011	Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein	130
CB-8705	Genetic and Biochemical Analysis of Cell Surface Function	134
CB-8010	The Morphologic Mechanisms of Motility and Malignant Transformation in Cultured Cells	137
CB-8012	Adenylate Cyclase Activity of Transformed and Normal Fibroblasts	141
CB-8709	Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth	145
CB-8700	Studies on the Regulation of Gene Expression in Bacterial and Animal Cells	148
CB-8006	Control of Gene Expression in *E. coli* K12 Bacteriophages λ and P22	152
CB-8701	An Examination of a Variety of Nucleic Acid Structural and Functional Relationships Involved in the Regulation of Cellular and Viral Gene Expression	156
CB-8711	Mechanism of Site-Specific Recombination in Bacteriophage Lambda	162
CB-8710	DNA Replication *In Vitro* Catalyzed by *E. Coli* Proteins	165
CB-8702	Endocytosis in the Thyroid Gland	168
CB-8703	Thyroid Hormone Synthesis and Storage	171

CB-8704	Production and Properties of Transplantable Thyroid Tumors	173
CB-8706	Mouse Mammary Tumor Virus - Host Cell Interaction	176
CB-8707	The Relationship of Genetic and Non-Genetic Factors in Mouse Mammary Tumorigenesis	180
CB-8708	Modulation of Differentiated Functions in Choriocarcinoma Cells in Culture	183

Laboratory of Pathophysiology

Summary Report		187
Project Reports:		
CB-08224	Arrest of Synthesis of Proteins during Regression of Hormone-Dependent Mammary Adenocarcinoma MTW9	193
CB-08213	Glucose-6-Phosphate Dehydrogenase Isozymes in Growing and Regressing Rat Mammary Carcinomas	195
CB-08223	Estrogen Binding Studies in Ovarian Dependent and Independent Rat Mammary Carcinoma	197
CB-05216	Role of Cyclic Nucleotide on In Vivo Tumor Growth Inhibition	200
CB-08222	Interrelationship of Cyclic Nucleotide and Steroid Hormone in Growth Control	205
CB-05219	In Vitro Simulation of Hormone-dependent Mammary Tumor Regression	209
CB-08205	Application of the Artificial Capillary Culture Technique to Polypeptide Hormone Production	213
CB-08230	Hormonal Control of Mammalian Follicular Maturation and Oogenesis	216
CB-08218	Purification and Characterization of Rat α-Lactalbumin	219
CB-08219	Isolation and Purification of Rat α-Lactalbumin and Casein m-RNA and their cDNA Probes	221

CB-08217	Synthesis of Mammary Specific Proteins - Casein and α-Lactalbumin - in Mammary Carcinoma Cells	223
CB-08231	Synthesis of α-LA and Casein mRNA, during Gestation and Lactation of Rat Mammary Gland, and in Rat Mammary Carcinomas	225
CB-08226	The Role of Thyroid Hormones in Growth and Differentiation of Mouse Mammary Glands	227
CB-08215	Angiogenesis as a Marker of Neoplastic Transformation	231
CB-08227	Relationship between Temperature and Blood Supply or Oxygen and Glucose Consumption by Mammary Carcinomas	233
CB-08220	Adenylation of Basic Cellular Proteins	235
CB-08221	Control of Histone H1 Dimer Poly(ADP-Ribose) Complex Formation by Poly(ADP-Ribose) Glycohydrolase	237
CB-05211	Modification of Histone H1 by Poly(ADPR) in Hela Cells	240
CB-08229	Role of Dietary Lipids in Increased Incidence of Mammary Cancer	243
CB-08210	Behavior of Ribosomes, Polysomes and Ribosomal Subunits in Non-dividing Human Cells and Changes Related to Onset and Cessation of Normal Cell Growth	246
CB-08212	Synthesis, Utilization and Degradation of Messenger RNA in Human Lymphocytes	250
CB-08211	Detection of Specific Messenger RNAs in Resting and Growing Human Lymphocytes	254
CB-08225	Structure and Function of Biological Membranes	255
CB-00380	Deoxyribose Containing Compounds in Normal and Cancer Tissues	258
CB-08228	Control of Synthesis of Serum Albumin and the Metabolism of Albumin Messenger RNA (mRNAalb.) in Normal Liver and Hepatic Carcinoma	262

CB-05205	Studies on Biochemical Mechanisms for Control of Nucleic Acid Synthesis	264
CB-00306	Modification of Amino Acids in Search of Possible Anti-tumor Agents	269
CB-05221	Comparison of Nucleoproteins of Normal Animal Tissues with Neoplastic Tissues	274
CB-00919	Cytochemical Features in Cancer	276
CB-00944	Total Metabolism of Cancer Cachexia	278
CB-00942	Effects of Gamma Irradiation on Nucleic and Proteins: Interactions of Cancer Chemotherapy Agents and Radiation	283
CB-00941	Immunogenetic Factors Affecting Bone Marrow Transplantation in Irradiated Mice	289
CB-00936	Repair of Sublethal Radiation Injury in Mice	294

Laboratory of Theoretical Biology

Summary Report 297

Project Reports:

CB-08300	SAAM Computer Program	301
CB-08301	Modeling Theory and Applications	303
CB-08303	Lipid Model Membrane Studies of Ion Transport Membrane Fusion and Cell Surface Mobility	305
CB-08306	Kinetic Modeling of Human Plasma Lipoprotein Metabolism	309
CB-08312	Computer Applications: Programming and Data Processing	312
CB-08317	Theory of Conformational Transitions in Polypeptides and DNA	315
CB-08320	Macromolecular Conformations	317
CB-08322	Physical Chemistry of Lymphocyte Activation	319
CB-08323	Quantitation of Immunochemical Assays	322

CB-08325	Compartment Modeling of Complex Systems	324
CB-08326	Dynamical System Theory in Biology	326
CB-08327	Mathematical Methods for Analysis of Large Scale Metabolic Networks	328
CB-08329	Mathematical Modeling of Renal Medullary Function	330
CB-08330	Mathematical Models of Carcinogenesis	332
CB-08331	Glucose-Insulin Kinetics Modeling of Carbohydrate Metabolism	335

Immunology Branch

Summary 339

Project Reports:

CB-05003	In Vitro Tests of Immunity in Patients with Melanoma	342
CB-05006	Lymphocyte Cell Surface Antigens, Normal and Neoplastic	345
CB-05011	Rhesus Monkey Histocompatibility Studies	353
CB-05016	Cell Mediated Cytotoxicity	356
CB-05017	Biological Significance of HLA Antigens	359
CB-05018	Killer Lymphocyte Damage to Target Cell Membranes	363
CB-05021	Antigens Determined by the Major Histocompatibility Locus	366
CB-05023	Transplantation Antigens of Swine	372
CB-05033	Immunotherapy of Human Cancer	376
CB-05034	Characterization and Isolation of Human Lymphocyte Subpopulations	379
CB-05035	Characterization of the Lymphocyte Receptor for Complexed Immunoglobulin	382

CB-05036	Genetic Control of the Immune Response to Staphylococcal Nuclease	386
CB-05038	Cell-Mediated Immunity to TNP-Modified Syngeneic Lymphocytes in Mice	391
CB-05043	AKR Leukemic Lines with T and B Cell Characteristics	396
CB-05045	Cell-Mediated Immunity to Nitrophenyl-Modified Syngeneic Lymphocyte Proteins	400
CB-05048	Attempted Isolation of Fc Receptor	402
CB-05049	F_1 Antiparental Cell-Mediated Lympholysis In Vitro	404
CB-05050	Studies on the Structure and Function of the Constant Portions of Immunoglobulins	407
CB-05051	Cell-Mediated Immunity to Spontaneously Arising Syngeneic Murine Tumors	412
CB-05052	Psychosocial Factors in the Prognosis of Melanoma	415
CB-05053	Resistance to AKR Leukemia	419
CB-05054	Mouse Macrophage Activation with BCG: Relationship to Anti-Tumor Activity In Vitro	423
CB-05055	Regulatory Influences on Cell-Mediated Immune Responses	426
CB-05056	Specific Removal of Histocompatibility Antigen-Reactive Cells	429
CB-05058	Immunoregulation by Antibody	433
CB-05059	Characterization of Antigen Specific T-Cell Factors	435
CB-05060	Physiological Role of Lymphocyte Surface Receptors	438
CB-05061	Specific Lymphocyte Adhesion to Target Surfaces	441
CB-05062	Application of Rapid Flow Microfluorometry to Cell Biology	443

CB-05063	Idiotype Specific T-Cell Responder	448
CB-05064	Genetic Control of the Immune Response In Vitro	451
CB-05065	Immunologic Function of Macrophages	453
CB-05066	Antibody Production to TNP-Modified Syngeneic Lymphocytes in Mice	456
CB-05067	Cell-Mediated Immunity to TNP-Modified Syngeneic Lymphocytes in Man	458
CB-05068	Immunology of Model Membrane Macromolecules	460
CB-05069	Expression of Ia Antigens on Functional Cell Subpopulations	463
CB-05070	The Effect of Tumor Cells in Regulating Cell Mediated Immunity	466
CB-05071	Effect of Sendai Viral Infection on the Immune Response	468
CB-05072	Affect of Viral Infection on the Serum Amyloid Precursor Protein (SAA)	470
CB-05073	Modification of Glycoproteins in Membranes of Animal Cells	472
CB-05074	Differential Susceptibility to Sendai Viral Infection	475
CB-05526	Cell Surface Changes in Spontaneously or by SV40 Transformed Mouse Cell Lines	478
CB-05544	Cell Surface Changes in Transformed Mouse Cell Lines	487

Laboratory of Immunodiagnosis

Summary Report 491

Project Reports:

CB-05557	Cellular Immune Responses in Experimental Tumor Systems	505

CB-08511	Characterization and Measurement of Soluble Antigens Associated with Neoplastic Disease	512
CB-08500	Immunodiagnosis, Immunotherapy and Immunological Monitoring in Human Cancer	520
CB-08522	Development and Use of Sensitive and Specific Assays of In Vitro Migration Inhibition for Monitoring Cellular Immunity to Tumor Antigens in Animals and Man	537
CB-08517	Cellular and Tumor Immunity in Experimental Animal Systems	550

Laboratory of Immunobiology

Summary Report 563

Project Reports:

CB-08525	Immunotherapy of Primary Autochthonous Cancer	573
CB-08526	Immunologic Approaches to the Treatment of Animals with Stage I Malignant Disease	576
CB-08527	Immunologic Approaches to Treatment of Animals with Stage II Malignant Disease	580
CB-08528	Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection	584
CB-08529	Immunotherapy with Defined Bacterial Components	587
CB-08550	Modification of Tumor Cells	590
CB-08551	Induction of Tumor Immunity by Chemotherapy	593
CB-08552	Mechanism of Complement Fixation and Action	596
CB-08553	Immune Cytolysis	599
CB-08554	Isolation and Study of Tumor-Specific Antigens	602
CB-08555	Detection of Complement Components on Nucleated Cell Surfaces	604
CB-08556	Ultrastructural Studies of Nucleated Cells Following Modification by Physical or Chemical Means	607

CB-08575 Inflammation 609

CB-08576 Immunological Mechanisms of Tumor Rejection 612

Laboratory of Cell Biology

Summary Report 617

Project Reports:

CB-03200 A Study of the Various Factors Influencing the Induction, Growth and Repression of Primary and Transplantable Neoplasms (Host and Tumor Cell Variables) 632

CB-03222 Genetic and Biochemical Mechanisms Controlling Protein Synthesis in Tumors 640

CB-03229 I. Chemical Structure of Immunoglobulin Molecules, Allotypes and Human Histocompatibility Antigens. II. Biochemical Characterization of Soluble Murine H-2 Antigens, Tumor Specific Antigens and Epoxide Hydrase 645

CB-04833 Biological Studies of Various Normal and Virus-Infected Malignant Cells 651

CB-04834 Viral Gene Expression in Normal Tissues and in Virus- or Chemically Induced Neoplasms 655

CB-05550 The Role of Retroviruses in Immunogenesis and Oncogenesis 659

CB-05552 Mammalian Cellular Genetics 664

CB-05553 Structure of Mouse Myeloma Proteins with Antibody Activity 668

CB-05596 Pathogenesis of Plasma Cell Neoplasia in Mice: Immunochemical Characterization of Antigen-Binding Myeloma Proteins: Genetic Basis of Immunoglobulin Diversity 672

CB-32555 Induction of Tumor Growth and Immune Response to Tumor Associated Antigens 678

Dermatology Branch

Summary Report		683
Project Reports:		
CB-3630	Morphologic and Biochemical Effects of Vitamin A and Analogs on Chick and Human Skin	688
CB-3632	Cutaneous Manifestations of Graft-Versus-Host Disease	693
CB-3638	Studies of DNA Repair in Normal Human Cells and in Cells from Xeroderma Pigmentosum Patients	696
CB-3643	Surgical Treatment of Darier's Disease (Keratosis Follicularis)	701
CB-3647	Scanning and Transmission Electron Microscopy of Leukocytes	705
CB-3648	T Cell Leukemias and Lymphomas: Morphologic and Functional Studies of the Neoplastic Cells	708
CB-3649	Enzymatic Control Mechanisms in Melanogenesis	713
CB-3650	Biochemical Characterization of Mammalian Melanosomes	716
CB-3652	Studies on Mechanisms of Cellular Immunity in Man	720
CB-3654	Applications of SEM to Soft Biological Tissues	727
CB-3656	Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments	731
CB-3657	Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases	736
CB-3658	Search for Animal Models for Genetic Skin Diseases	741
CB-3659	Therapy of Skin Cancer and Disorders of Keratinization	745
CB-3660	Transfection of Viral DNA of C-Type RNA Viruses	749
CB-3661	Human Papilloma Viruses	752

CB-3662 Factors Involved in the Elimination of Superficial Mycotic Infection in Guinea Pigs 755

CB-3663 Tumor Virus Expression In Vitro and In Vivo 758

Metabolism Branch

Summary Report 761

Project Reports:

CB-4002 Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction 775

CB-4003 Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria 784

CB-4004 Studies of Amino Acid Biosynthesis and Degradation 787

CB-4014 Studies of the Immune Response in Normal and Pathological States 791

CB-4015 Studies of Development and Function of Humoral and Cellular Immune Mechanisms 799

CB-4016 Mechanism of the Anabolic Action of Growth Hormone 806

CB-4017 Biology of the Immune Response 810

CB-4018 Study of Human Immune Defense Mechanisms and Its Control 816

CB-4019 Immunoregulatory Cells in Human Neoplasia 820

CB-4020 Genetic Control of the Immune Response to Natural Protein Antigens 827

Laboratory of Pathology

Summary Report 831

Project Reports:

CB-00853 Surgical Pathology and Postmortem Section 852

CB-00852 Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems 857

CB-00582 Chronic Mouse Cytomegalovirus Infection 861

CB-00585	Histological Evolution of In-Situ Carcinoma in the Vagina	863
CB-00591	Immunofluorescence Studies on Human Tumors	865
CB-00592	A Morphologic Investigation of Virus Induced Membrane Receptors	867
CB-00594	Pathology of Family Cancer Studies	869
CB-00504	An Electron Optical Investigation of Normal and Pathologic Tissue	871
CB-00505	Pulmonary Carcinogenesis: Testing and Examining the Mechanisms Through Animal Models	873
CB-00516	Comparative Studies on Neoplasms in Relation to Pathogenesis, Etiology and Diagnosis	880
CB-00521	Influence of Epithelial-Mesenchymal Interactions on Neoplastic Development	883
CB-00538	Phylogenetic Aspects of Neoplasia	889
CB-00540	Contractile Tissues in Organ Cultures of Mouse Submandibular Glands	894
CB-00560	Electron Probe and Scanning Electron Microscope	896
CB-00561	Collaborative Research and Other Activities	898
CB-00593	An Investigation of the Collagenous Connective Tissues and Elastin	900
CB-00533	Studies on Oncogenic DNA Viruses	902
CB-00534	Structure and Assembly of Virulent and Vaccine Polioviruses	904
CB-00535	Biologic and Virologic Characterization of Breast Cancer Cell Lines	906
CB-00517	Pathological Technology Section	908

CONTRACT PROGRAMS

		Page
IMMUNOLOGY CONTRACT PROGRAM		
Summary Report		911
Contract Research Summaries:		
Contract No. NO1-CB-		
64034	Cells Involved in Immune Response to Tumors	923
44008	Organization and Dynamics of Cell Surface Membrane Components	924
64067	Biochemistry of Normal and Tumor Cell Surface Antigens	925
64035	Cells Involved in Immune Response to Tumors	926
43924	Detection of Antigen-Binding Activity of Transplantable T-Cell Tumors	927
53957	Cell-Mediated Immunity to Epstein-Barr Virus	928
43988	Production and Distribution of H-2 Recombinant or Mutant Congenic Strains of Mice	929
43999	Specificity of Antigen-Binding Receptors on T Cells	930
43922	Organization and Dynamics of Cell Surface Membrane Components	931
43932	Mechanisms for Cell-Mediated Destruction of Tumor Cells	932
43985	Biochemistry of Normal and Tumor Cell Surface Antigens	933
33870	Effector Mechanisms in Tumor-Associated Immune Reactions	934
43921	Human Tumor-Associated Antigens and Corresponding Antibodies	935
64023	Cells Involved in Immune Response to Tumors	936
53959	Effects of Streptoccal Serum Factors on Lymphocyte Responses	937

43928	Identification, Quantitation and Characterization of Lymphocytes and Macrophages	938
43925	Serologic and Immunogenetic Investigation of Cell Surface Antigens	939
43927	Mechanisms of Lymphoid Cell Differentiation	940
53868	Characterization of Purified Thymic Products Promoting Lymphocyte Differentiation	941
64039	Immunogenicity of "Spontaneous" Animal Tumors	942
43931	Investigations of the Nature and Function of Immune-Related Cells in Tumor Masses	943
53876	Liposome Model System	944
64038	Immunogenicity of "Spontaneous" Animal Tumors	945
64036	Cells Involved in Immune Response to Tumors	946
63973	Role of the Macrophage in Tumor Resistance	947
23886	Facility for Supplying Immune-Related Cell Lines	948
33879	Supramolecular Organization of Normal and Tumor Cell Surfaces	949
44001	Investigations of the Nature and Function of Immune-Related Cells in Tumor Masses	950
53881	Techniques for In Vitro Sensitization of Human Lymphocytes	951
64031	Cells Involved in Immune Response to Tumors	952
53942	Human Tumor-Associated Antigens and Corresponding Antibodies	953
53935	Automated Systems for HLA Typing	954
43941	Recombinant Inbred Strains of Mice for Analysis of Ir-1, Rgv-1 and Linked Genes	955
43858	Nature of Interactions Between Tumor Cells and Immunoglobulins	956
43970	Selective Stimulation or Suppression of Humoral or Cellular Immunologic Responses	957

44000	Organization and Dynamics of Cell Surface Membrane Components	958
43971	Mechanisms of Lymphoid Cell Differentiation	959
44009	Biochemistry of Normal and Tumor Cell Surface Antigens	960
23883	Analysis of Serum Requirements for In Vitro Immunological Studies	961
43972	Genetic Control of Immune Responses in Relation to Cancer	962
43998	Selective Stimulation or Suppression of Humoral or Cellular Immunologic Responses	963
63978	Studies by Which Tumors Avoid Detruction by the Immune System	964
63977	T-Memory Cell Properties Primed to Histo-incompatible and Syngeneic Tumor Cells	965
64032	Cells Involved in Immune Response to Tumors	966
43923	Specificity of Antigen-Binding Receptors on T Cells	967
43997	Selective Stimulation or Suppression of Humoral or Cellular Immunologic Responses	968
64009	Immunogenicity of "Spontaneous" Animal Tumors	969
43987	Organization and Dynamics of Cell Surface Membrane Components	970
43986	Biochemistry of Normal and Tumor Cell Surface Antigens	971
64027	Subpopulation of Specific Sera	972
64033	Cells Involved in Immune Response to Tumors	973
33854	Granulocyte and Macrophage Proliferation Control in Leukemia and Related Neoplasms	974
63992	Transfer Factor and Delayed Type Hyper-sensitivity in the Mouse	975

23889	The Role of T and B Lymphocytes in Tumor Immunity	976
74154	Detection and Development of H-2 Recombinant and Mutant Strains	977
63982	Studies of the Mechanism by Which Tumors Avoid Destruction by the Immune System	978
63979	Techniques for In Vitro Sensitization of Human Lymphocytes	979
43930	Studies of the Mechanisms by Which Tumors Avoid Detruction by the Immune System	980
53926	Characterization of Purified Thymic Products Promoting Lymphocyte Differentiation	981
43929	Selective Stimulation or Suppression of Humoral or Cellular Immunologic Responses	982
43994	Selective Stimulation or Suppression of Humoral or Cellular Immunologic Responses	983
63993	Detection of Antigen-Binding Activity of Transplantable T-Cell Tumors	984
43926	Purified Thymic Products and Other Agents Promoting Lymphocyte Differentiation	985
74119	Clinical Evaluation of Immunodiagnostic Tests for Cancer	986
53955	Purification of Human Tumor-Associated Antigens	987
43887	Evaluation of Assays for Circulating Tumor-Associated Antigens: CEA	988
43888	Evaluation of Assays of Circulating Tumor-Associated Antigens: AFP and HCG	989
33858	Tumor-Specific Antigens in Diagnosis and in the Management of Cancer Patients	990
53901	Development of Macrophage Electrophoretic Mobility Assay for Malignant Disease	991
43965	New In Vitro Techniques to Evaluate Cell-Mediated Immunity to Intact Tumor Cells	992

64071	Diagnostic Application of Human Tumor-Associated Antigens	993
43889	Human Tumor-Associated Antigens of the GI Tract Other Than CEA and α-Fetoprotein	994
23879	Blood Sample Collection Bank as Reservoir for Evaluation of Cancer Detection Tests	995
43953	Detection of Antibodies Against Autologous Human Tumor Cells	996
53954	Purification of Human Tumor-Associated Antigens	997
33914	Collection of Sera from Populations with High Cancer Risk	998
43958	Human Tumor-Associated Antigens of the GI Tract Other Than CEA and Alpha-Fetoprotein	999
53934	Evaluation of an Isomeric Species of Carcinoembryonic Antigen (CEA-S)	1000
43277	Isolation and Characterization of Soluble Human Tumor (CEA)-Specific Antigens	1001
53899	Purification of Human Tumor-Associated Antigens	1002
53952	Detection of Circulating Antigen-Antibody Complexes in Cancer	1003
43885	Improvement of Methods for Isolation and Preservation of Lymphocytes	1004
43964	Isolation and Characterization of Human Peripheral Blood Mononuclear Cells	1005
43854	Isolation and Tissue Culture of Human Tumor Cells	1006
64021	Diagnostic Application of Human Tumor-Associated Antigens	1007
53894	Role of Antibody-Dependent Cell-Mediated Cytotoxicity in Tumor Immunity	1008
43989	Endoglycosidases Capable of Releasing Oligosaccharides from Glycoproteins	1009
64070	Diagnostic Applications of Human Tumor-Associated Antigens	1010

53893	Detection of Circulating Antigen-Antibody Complexes in Cancer	1011
43860	Development of Immunodiagnostic Tests for Cancer	1012
53900	Isolation and Characterization of Human Peripheral Blood Monocytes	1013
43891	Animal Models for Circulating Tumor-Associated Antigens for Diagnosis of Cancer	1014
64072	Diagnostic Application of Human Tumor-Associated Antigen	1015
33915	Collection of Sera from Populations with High Cancer Risk	1016
43881	Immunofluorescent Detection of Antibodies Against Autologous Human Tumor Cells	1017
43893	New In Vitro Techniques to Evaluate Cell-Mediated Immunity to Intact Tumor Cells	1018
64068	Diagnostic Applications of Human Tumor-Associated Antigens	1019
43883	Isolation and Characterization of Human Peripheral Blood Mononuclear Cells	1020
53896	Antibodies to Human Organ- or Tissue-Associated Antigens	1021
53956	Development of Macrophage Electrophoretic Mobility Assay for Malignant Disease	1022
63990	Detection of Circulating Antigen-Antibody Complexes in Cancer	1023
43890	Search for New Human Tumor-Associated Antigens in Carcinoma of the Lung	1024
43882	Early Detection of Antibodies to Carcinomas in Experimental Animal Models	1025
43859	Biochemistry and Diagnostic Use of Human Tumor Antigens	1026

(NO1-CP-)

65783	Synthesis of Cord Factor-Like Components	1027
23243	Production and Detection of Antibodies to Chemical Carcinogens and Other Small Molecules	1028
43354	Tumorigenesis in Tolerant Guinea Pigs	1029
45612	Study Effects of Carcinogens on the In Vitro Synthesis of Complement Components	1030
65744	Induction of Colonic Tumors in Guinea Pigs	1031
33313	Response of Peripheral Blood Monocytes to Chemotactic Factors	1032
65746	Procurement of Sulfolipids from Mycobacterium tuberculosis Strain H37RV	1033
53329	Chemical Carcinogenesis and Immunity	1034
55679	Chemical Carcinogenesis and Immunity	1035
33200	Induction of Malignant Melanoma in Guinea Pigs	1036
65778	Studies on an In Vivo/In Vitro System as a Potential Bioassay	1037
43353	Development of In Vitro Methods for the Detection of Cell-Mediated Immunologic Reactivity to Chemical Carcinogens	1038

NO1-CB-

53940	Intrapleural BCG after Primary Surgery for Lung Cancer	1039
43949	Augmentation of Tumor-Specific Immunity with Ribonucleic Acid (RNA)	1040
64048	Induced Thyroiditis as an Immunotherapeutic Approach to Thyroid Cancer	1041
64061	Studies of Immune Stimulants in Patients Receiving Radiation Therapy	1042
64018	Immunotherapeutical Trials with Human Tumors	1043
64001	Immunotherapy in Outbred Cat Lymphomas and Leukemias	1044

64007	Tumor-Specific Active Immunotherapy of Squamous Cell Carcinoma of the Lung	1045
64050	Therapy of Murine Leukemias Using Lymphoid Cells Sensitized to Leukemia Antigens	1046
02208	Preclinical Studies on Tumor Protective Activity of MER	1047
53870	Preparation and Distribution of Rabbit Serum Complement	1048
43872	The Immunological Stimulant Properties of Amphotericin B	1049
43954	Test Device with Standardized Antigens to Assay Delayed Hypersensitivity	1050
64064	Squamous Cell Carcinoma of the Lung Treated by Resection or Radiotherapy	1051
53874	Phase I Study of Effects of Immune Stimulants on Human Immune Responses	1052
33888	Immunotherapy of Human Cancer	1053
64003	Immunotherapy in the L_2C Guinea Pig Leukemia	1054
43877	Immunotherapy: Mechanism of Action of Immunopotentiators	1055
43879	Chemoimmunotherapy of Acute Myelocytic Leukemia	1056
64043	Immunotherapy in Fibrosarcomas of Chickens	1057
53936	Qualitative Assays of Monocyte-Macrophage Function	1058
43878	Transfer Factor Therapy of Sarcoma	1059
53937	New Approaches to Immunotherapy	1060
43864	Animal Models for Evaluation of Therapy with Neuraminidase-Treated Tumor Cells	1061
43873	Role of Macrophages in the Immune Response to Tumors	1062
33891	Animal Models for Treatment of Minimal Residual Systemic Tumor	1063

(NO1-CP-)

33373	Immunotherapy of Accessible Neoplasms and Analysis of Immune Status In Vivo	1064

NO1-CB-

33896	Macrophage Activation in Tumor Immunity and Immunotherapy of Rat Mammary Tumor	1065
53869	Quantitative Assays of Monocyte-Macrophage Function	1066
64057	Activated Macrophages as Immunotherapeutic Agents	1067
43951	Effect of Transfer Factor on Virus-Induced Tumors in Marmoset Monkeys	1068
43874	Role of Circulating Tumor Antigens	1069
53970	Phase I Study of Effects of Immune Stimulants on Human Immune Response	1070
64062	Evaluation of Immunotherapy with Tumor Preparation in Man	1071
64051	Therapy of Tumors in Mice with Tumor Necrosis Factor (TNF)	1072
53873	Evaluation of C. parvum in Disseminated Carcinoma of the Breast	1073
53875	Levamisole as a Therapeutic Adjunct in Squamous Cell Carcinoma of Head and Neck	1074
64055	Immunotherapy of Herpes Virus Lymphomas in Marmosets	1075
63988	Immunotherapy with In Vitro Lymphocyte Sensitization	1076
64053	Modified Tumor Cell Membranes as Immunotherapeutic Agents	1077
23221	Study of Microorganisms as Inhibitors of Tumor Cell Growth	1078
53885	The Production of an Improved BCG Vaccine	1079

53941	Specific and Nonspecific Immunotherapy as an Adjuvant to Chemotherapy	1080
53939	Evaluation of C. parvum in Advanced Cancer of the Breast and Lung	1081
64076	Human Melanoma: Evaluation of BCG Immunotherapy	1082
64004	Immune Status of Patients and Effects of Immunostimulants on Radiation Therapy	1083
64006	Active Specific Immunotherapy in Acute Myelogenous Leukemia	1084
53933	Assessment of Tumor-Specific Immunity in Osteosarcoma Patients	1085
43857	Human Lung Cancer -- Evaluation of BCG Therapy	1086
43946	Immmunotherapy: Role of Circulating Tumor Antigens	1087
43948	Role of Macrophages in Immune Response to Tumors	1088
23885	Immunotherapy of Cancer in Man	1089
64002	Study of Mycobacterium phlei in Lung Cancer, Hypernephroma and Malignant Melanoma	1090
64042	Immunotherapy of Syngeneic Tumors in Wistar Rats	1091
43967	Hapten Treatment of Cancer	1092
64054	Evaluation of Immunity Induced by Drug (DTIC)-Mediated Tumor Antigens	1093
64044	Immunotherapy of C_3H Murine Mammary Carcinomas	1094
64058	Immunotherapeutic Studies with Lymphokine 1788	1095
64049	Antibody-Drug Conjugates in Immunotherapy	1096
64005	Immunotherapy of Mouse Tumors Using Immuno-responsive Cells Sensitized In Vitro	1097
43865	Development of Therapy with Neuraminidase-Treated Tumor Cells	1098
64024	Maintain an Animal Holding Facility and Provide Attendant Research Services	1099

53890	NCI Histocompatibility Typing Center	1100
64022	Cutaneous Hypersensitivity Antigen Reactions	1101
53888	Systems Analysis and Informational Service in Registries of Clinical Protocols	1102
63975	Measurement of Immunological Reactivity to Human Cancer	1103
63987	Biologic Studies of Solubilized Tumor Antigens	1104
74093	Studies of the Immune Response of Mice and Rats to Tumor Antigens	1105
53916	Melanoma Cell Vaccine and In Vitro Assays for Humoral and Cellular Cytotoxicity	1106
64016	Rhesus Monkey Histocompatibility Studies	1107
92141	Induction, Transplantation, and Preservation of Plasma Cell Tumors in Mice	1108
63976	National Cancer Institute Immunodiagnostic Reference Center	1109
53912	Preparation of Reagent Antisera and Antigens	1110

BREAST CANCER TASK FORCE PROGRAM

Summary Report 1111

Contract Research Summaries:

Contract No. NO1-CB-		
23872	Ultrasound Mammography	1114
23871	Immunological Studies of Human Mammary Carcinoma	1115
44011	Xeromammography vs. Film Mammography -- A Comparative Study	1116
44012	Xeromammography vs. Film Mammography -- A Comparative Study	1117
74087	Immunologic Cross Reactions Between Breast Cancer and Mammary Tumor Virus	1118
64047	Clinical Evaluation of Computerized Tomographic Mammography	1119
64041	Breast Diagnosis: Quantitative Imaging by Ultrasound	1120
23869	Tissue Culture Lines from Patients with Breast Cancer	1121
43993	Xeromammography vs. Film Mammography -- A Comparative Study	1122
23867	Breast Cancer Tissue Supply	1123
74097	Prognostic Factors for Disseminated Cancer in Patients with Early Breast Cancer	1124
74206	Longitudinal Studies of Biologic Markers in Breast Cancer Patients	1125
53855	Evaluation of Serum LDH Isoenzymes in Women with Breast Tumors	1126
71465	Steroid Excretion Study in Patients with Breast Cancer	1127
80704	Pathology Laboratory for Cooperative Clinical Studies	1128

53853	Biochemical Analysis of Human Breast Cyst Fluid (BCF)	1129
74086	Longitudinal Studies of Biologic Markers in Breast Cancer Patients	1130
23231	Primary Breast Cancer Study Patient Input (Follow-up)	1131
33882	Analysis of Breast Secretions	1132
43869	Screening for Breast Cancer by Electronic Infrared Pattern Recognition	1133
23232	Primary Breast Cancer Study Patient Input (Follow-up)	1134
43871	Fibrocystic Disease and Its Relationship to Breast Cancer	1135
74098	Risk Associated with In-Situ Carcinoma and "Precancerous" Mammary Hyperplasias	1136
43898	Blood Steroid Sulfate Levels as Diagnostic Parameters in Breast Cancer	1137
74099	Estrogen Replacement after Pre-Menopausal Oophorectomy and Breast Cancer Risk	1138
53883	Correlations Between Dietary Factors and Pre- and Postmenopausal Breast Cancer	1139
74102	Epidemiology of Benign Breast Disease	1140
33901	Breast Cancer Family Resource	1141
44003	Genetic Polymorphisms in High- and Low-Risk Breast Cancer Families	1142
63996	Epidemiologic Characteristics of Pre- and Postmenopausal Breast Cancer	1143
74101	Endocrine Events at the Time of First Pregnancy	1144
74100	Pathogenetic Models of Malignant and Pre-Malignant Disease of the Breast	1145
43973	A Study on Familiality of Breast Cancer in Iceland	1146

53854	Correlations Between Morphology and Epidemiology of Breast Cancer	1147
44002	Biochemical and Physiological Investigations Based on Familial Genetic Patterns	1148
43940	Genetic Polymorphisms in High- and Low-Risk Breast Cancer Families	1149
74104	Relation of Intestinal Flora to Breast Cancer in Humans	1150
74103	Epidemiology of Minimal Breast Cancer in Initially Asymptomatic Women	1151
63997	Epidemiologic Characteristics of Medullary and Lobular Breast Cancer	1152
53968	Correlations Between Morphology and Epidemiology of Breast Cancer	1153
63998	Epidemiologic Characteristics of Pre- and Postmenopausal Breast Cancer	1154
74202	Epidemiology of Benign Breast Disease	1155
53884	Role of Dietary Factors and Non-Contraceptive Estrogens in Breast Cancer	1156
44004	Genetic Polymorphisms in High- and Low-Risk Breast Cancer Families	1157
74107	Relationship of Thyroid Function to Growth of Mammary Tumors	1158
53904	Effects of Nucleic Acids on the Biological Properties of Mammary Cancer	1159
43907	Role of Stroma in Neoplasia of the Mammary Gland	1160
74189	Effects of Neonatal Exposure to Estrogen and Progestin on the Mammary Gland	1161
23859	Role of Prolactin in Breast Cancer	1162
33907	Pathological History of the Mammary Gland in Pseudohermaphroditic Rats and Mice	1163

23857	Protein Nucleic Acid Interactions in Transcription of Normal and Malignant Cells	1164
74207	Prevent the Formation and Progression of Mammary Gland Preneoplastic Lesions	1165
33883	The Response of the Embryonic Mammary Gland to Androgenic Hormones	1166
74109	Influence of Mammary Carcinoma Cells on the Formation and Absorption of Collagen	1167
74094	Growth and Passage of Primary Cultures of Normal Mammary Epithelial Cells	1168
74175	Animal Mammary Tumors and Human Cell Bank Facility	1169
63983	Identification of Mammary Tissues	1170
74090	Prevent the Formation and Progression of Mammary Gland Preneoplastic Lesions	1171
33910	Biochemical Means by Which Effector Molecules Bind to Mammary Cell Surfaces	1172
63984	Glycoproteins of the Mammary Cell Surface	1173
33898	Cultivation of Normal and Malignant Human Mammary Tissue	1174
23863	Isolation of Prolactin Cells from Human and Rat Adenohypophysis	1175
23865	Studies on Basic Biological Events in the Pathogenesis of Mammary Cancer	1176
23864	Studies on the Prevention of Metastasis in Mammary Cancer	1177
74105	Change in Mammary Sensitivity to Carcinogens Related to Host Physiological State	1178
43905	Mammary Gland Responsiveness to Multiple Hormones	1179
43904	Effects of Nucleic Acids on the Biological Properties of Mammary Cancer	1180

53903	Role of Stroma in Growth of Neoplasms of the Mammary Gland	1181
33905	Biochemical Means by Which Effector Molecules Bind to Mammary Cell Surfaces	1182
23875	Control of DNA Synthesis in the Mammary Gland	1183
33856	Study Breast Cancer Transplantability in Nude-Athymic Mice	1184
64074	Purification of Estrogen Receptor in Tangible Amounts	1185
63981	Microcirculation and Molecular Transport in Mammary Carcinomas	1186
43866	Mammary Gland Responsiveness to Multiple Hormones	1187
74188	Growth and Passage of Primary Cultures of Normal Mammary Epithelial Cells	1188
74091	Effects on Neonatal Exposure to Estrogen and Progestin on the Mammary Gland	1189
43908	Neoplastic Potential of Preneoplastic Lesions of the Human Mammary Gland	1190
23873	Biochemical Nature of Hormone Dependency in Breast Cancer	1191
74089	Effect of Mammary Carcinoma Cells on the Vascular Endothelium	1192
63980	Osteotropism of Mammary Carcinoma Netastasis	1193
63985	Structure Function Relationship of Prolactin Interactions in Mammary Gland Cells	1194
53905	Study of Mammary Gland Responsiveness to Multiple Hormones	1195
74108	Methylation of Transfer RNA in Normal and Neoplastic Mammary Tissue	1196
33880	Studies of Isoenzymes in Normal, Preneoplastic and Neoplastic Breast Tissues	1197

23862	Biochemical Mechanism of Endocrine-Induced Breast Cancer Regression	1198
43906	Alteration of Mammary Neoplastic Cells by Manipulation of Cellular Environment	1199
63986	Differentiation of Mammary Epithelial Cells	1200
64069	Glycoproteins of Mammary Cell Surface	1201
33908	Isolation and Characterization of Mammary Epithelial Cell Plasma Membrane	1202
33919	Role of 3', 5'-cAMP and Related Enzyme Systems in Mammary Neoplasia	1203
43899	Cell Kinetics of Breast Cancer	1204
75083	Methods to Predict Chemotherapy Sensitivity	1205
43990	Therapy for Stage II or Stage III Carcinoma of the Breast	1206
53917	Therapy of Patients with Stage II or Stage III Carcinoma of the Breast	1207
33899	Surgical Adjuvant Chemotherapy in Patients with Breast Cancer	1208
43914	Predictive Transplantable Animal Mammary Tumor Models	1209
74208	Methods to Predict Chemotherapy Sensitivity	1210
74140	Biomedical Computing Software Services	1211
33903	Cell Kinetics of Breast Cancer	1212
33861	New Techniques in Cell Kinetics of Breast Cancer	1213
53851	Suppression of Endocrine Function by Systemic Agents for Breast Cancer Therapy	1214
43900	Investigation of Steroid Sulfation and Estrogen Binding in Human Breast Cancer	1215
43991	Hormone Manipulation Plus Chemotherapy in Breast Cancer Patients	1216
74110	Occult Breast Cancer Metastases in Axillary Lymph Nodes	1217

43917	Therapy of Patients with Stage II Carcinoma of the Breast	1218
43969	Prediction of Hormone Dependency in Human Breast Cancer	1219
64000	Endocrine Therapy Plus Chemotherapy in Patients with Breast Cancer	1220
74203	Methods to Predict Chemotherapy Sensitivity	1221
23876	Primary Breast Cancer Group (NSABP)	1222
74204	Methods to Predict Chemotherapy Sensitivity	1223
74205	Methods to Predict Chemotherapy Sensitivity	1224
43867	Estrophile Binding and Estrophile Proteins in Human Breast Cancer	1225

CANCER DIAGNOSIS CONTRACT PROGRAM

Summary Report		1227
53858	Development of a Computerized Transaxial X-ray Reconstruction System	1230
64010	Standard Protocol for Evaluation of Imaging Techniques in Cancer Diagnosis	1231
43910	Clinical Evaluation of CTT in Brain Tumor Diagnosis	1232
53974	Fabrication and Evaluation of Computerized Transaxial X-ray Reconstruction System	1233
43980	Clinical Evaluation of CTT in Brain Tumor Diagnosis	1234
50304	Algorithms for Computerized Transaxial Nuclide Reconstruction	1235
40302	Exploration of the Use of a Proton Beam in Tissue Densitometry	1236
43981	Clinical Evaluation of CTT in Brain Tumor Diagnosis	1237
53913	Algorithms for Computerized Transaxial Nuclide Reconstruction	1238

43983	Clinical Evaluation of CTT in Brain Tumor Diagnosis	1239
43982	Clinical Evaluation of CTT in Brain Tumor Diagnosis	1240
53857	Data Base for Testing Algorithms for Computerized Transaxial	1241
53860	Algorithms for Computerized Transaxial X-ray Reconstruction	1242
64012	Radioisotope Surface Markers and Detectors for Endoscopic Techniques	1243
64073	Development of Immunoglobulins Labeled with Gamma-emitting Radioisotopes	1244
64011	Development of Immunoglobulins Labeled with Gamma-emitting Radioisotopes	1245
53859	Algorithms for Computerized Transaxial Nuclide Reconstruction	1246
43974	Methods of Bowel Preparation Preparatory to Barium Enema or Colonoscopy	1247
53863	Determination of Optimal Frequency of Screening Strategies	1248
63994	Screening for Medullary Carcinoma of the Thyroid Using Calcitonin Radioimmunoassay	1249
43903	Hormone Markers for the Detection and Diagnosis of Cancer	1250
45037	Lung Cancer Control: Detection and Treatment	1251
92172	Lung Cancer -- Early Detection, Localization, and Therapy	1252
53886	Lung Project for Detection and Localization of Early Lung Cancer	1253
23854	Usefulness of Carcinoembryonic Antigen in the Diagnosis of Bowel Cancer	1254
43895	Detection of Pancreatic Cancer at Early or Small Stage Prior to Metastasis	1255

43975	Detection of Pancreatic Cancer at Early or Small Stage Prior to Metastasis	1256
45007	Lung Cancer -- Early Detection, Localization and Therapy	1257
53907	Determination of Optimal Frequency of Screening Strategies	1258
64013	Early Detection of Ovarian Carcinoma in Asymptomatic Women	1259
43977	Biochemical Markers or Enzyme Changes That May Presage the Presence of Cancer	1260
64028	Early Detection of Ovarian Carcinoma in Asymptomatic Women	1261
53865	Improving X-ray Imaging, Contrast, and Sharpness While Retaining Film Resolution	1262
43976	Detection of Pancreatic Cancer at Early or Small Stage Prior to Metastasis	1263
43868	Statistical Center for Cooperative Lung Cancer Groups	1264
43968	Detection of Small Amounts (over 7-10 ml/24 hours) of Human Blood in Human Feces	1265
53862	Screening Technique for Blood in Stool to Detect Early Cancer of Bowel	1266
64029	Development and Serum Isozyme Test for Human Cancer Patients	1267
53864	Experiment to Assess the Impact of Multi-site Screening on Total Cancer Mortality	1268
53914	Improvement of X-ray Imaging by Elimination of Scatter While Retaining the Resolution	1269

CYTOLOGY AUTOMATION CONTRACT PROGRAM

Summary Report		1271
	Development of New Methods of Single Cell Separation	1273
60311	Application of Flow System Methods of Cell Analysis and Sorting to Cancer Screening	1274
64015	Vaginal-Cervical Sample Sources	1275
	Application of Digital Image Processing Techniques to Cytology Automation	1276
43945	Evaluation of Markers for Gynecologic Specimens	1277
40300	New Stains and Other Optical Markers Useful for Gynecologic Specimens	1278
43912	Development of Hematoxylin Substitutes for Staining of Tissues and Exfoliated Cells	1279
43963	Methods for Obtaining Monodisperse Suspensions of Cytology Specimens	1280
53915	Furnishing a Cytoscreener and Technician for Testing Purposes	1281
43962	New Stains and Other Optical Markers for Clinical Specimens in Suspension	1282
	Immunologic Markers Applicable to Cytology Automation	1283
43984	Methods for Obtaining Monodisperse Cells and Markers for Gynecologic Specimens	1284
43943	Markers for Detection of Abnormal Cervical Cells by Optical Methods	1285
	Application of Digital Image Processing Techniques to Cytology Automation	1286
43955	Methods for Obtaining Monodisperse Preparations of Cytology Specimens	1287

43937	Vaginal and Cervical Cell Sample Sources for Cytology Automation	1288
	Immunologic Markers Applicable to Cytology Automation	1289
43960	Evaluation of Stains Useful for Gynecologic Specimens	1290
33873	Image Processing for Development of Automated Cell Recognition System	1291
	Immunologic Markers Applicable to Cytology Automation	1292
33862	Slit-Scan Technique as Cancer Prescreening Automated System in Cytology	1293
53930	High-Resolution Fluorescence Measurements and Quantitative Study of Fluorochromes	1294
53867	Evaluation of Engineering and Biological Operations of NLC Cytoscreener	1295
43746	Pre-Clinical Studies of Chemotherapeutic Studies for Mycosis Fungoides	1296
(YO1-CB-)		
80047	Computer Services	1297
NO1-CB-		
63927	A Screening System for Topical Chemotherapy of Mycosis Fungoides	1298
53866	Administrative Support Services	1299
(YO1-CB-)		
60307	Development of a Version of the SAAM Program for the Control Data (CDC) Computer	1300
NO1-CB-		
43912	Development of Hematoxylin Substitutes for Diagnostic Staining of Tissues and Exfoliated Cells	1301

64079	Preparation and Analysis of Cell Surface Protein (CSP) Fractions	1302
63932	Radioimmunoassay of Immunoglobulin Molecules	1303
43236	Detection and Expression of Tumor Viruses	1304
33887	Small Animal Holding Facility	1305
33874	A Study of Phylogenetic Aspects of Neoplasia	1306

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

SUMMARY REPORT OF THE DIRECTOR

July 1, 1976 Through September 30, 1977

INTRODUCTION

The Division of Cancer Biology and Diagnosis consists of both intramural and collaborative research programs in the biology and diagnosis of cancer. The Division also coordinates the activities of the Breast Cancer Task Force and the Lung Cancer Screening Program, and manages a collaborative program in immunology. A close coordination is maintained with other divisions of NCI in areas of common interest.

The intramural research program consists of three broad areas: biology, immunology, and clinical research. A group of laboratories performing basic research in cancer biology includes the Laboratories of Biochemistry, Pathophysiology, Molecular Biology, and Theoretical Biology. Four units, Immunology Branch and the Laboratories of Immunobiology, Immunodiagnosis and Cell Biology, together with a sizeable contract program, are assigned to the Associate Director for Immunology. Three clinical branches work in the fields of Dermatology, Metabolism, and Pathology. A fourth organizational element, under the Associate Director for Collaborative Research, manages the three major contract programs: Cancer Diagnosis, Breast Cancer Task Force, and Cytology Automation.

Organizationally, the Division underwent few changes last year. The Laboratory of Immunobiology was transferred from the Division of Cancer Cause and Prevention to this Division under the Associate Director for Immunology. The Image Processing Unit was transferred to the Laboratory of Pathology where its activities are coordinated with the Quantitative Cytology Section and the Cytology Automation Program.

As noted in the introduction, much of the cancer biology research other than tumor immunology and clinical research is carried out in four laboratories: Biochemistry, Molecular Biology, Pathophysiology and Theoretical Biology. It is almost exclusively an intramural effort with but a few contracts supporting it with limited service.

The Laboratory of Biochemistry consisting of six sections is directed by Dr. R. F. Goldberger. During the reporting period, the Section of Cell Biology and Oncogenesis was transferred to the Division of Cancer Cause and Prevention and a new section is being organized in its place--the Developmental Biology Section.

The work in the Section of Biochemistry of Gene Expression ranged from studies of the control of gene expression in eukaryotic cells to the effect of steroid hormones on enzyme induction and cell functions, both areas finding clinical applications. Cell-free translation of mRNAs for tyrosine aminotransferase and prolactin is being developed in an attempt to estimate functional levels of corresponding messenger RNAs during induction by hormones in cell systems.

The Biosynthesis Section works on the relationship between cell function and organization at the molecular level. Some of the topics under investigation include collagen synthesis, cellular interaction in the immune response, and genetic information associated with virus-like type-A particles. An improved method was developed for rapid purification of DNA polymerase alpha from mouse myeloma. Another aspect of this program is the characterization of protein factors capable of modifying the activity of isolated polymerases. Examination of the ultrastructure of C-reactive protein showed it to consist of 5 protomers arranged in cyclic symmetry -- a rare phenomenon.

The studies in the Cellular Regulation Section are oriented toward the regulation of gene expression at the molecular level. The studies involve the following steps: isolation of specific messenger RNA, identification of this RNA, purification, use of purified RNA for synthesis of complementary DNA and finally its application as a probe for hybridization studies of in vitro transcripts from purified components with RNA polymerase and chromatin. Using this outline, work has been initiated on the induction of vitellogenin synthesis by estrogen in rooster liver.

Enzymes involved in synthesis and degradation of nucleic acids are investigated in the Nucleic Acid Enzymology Section. Considerable effort was devoted to ultrapurification of various proteins with the result that several conflicting views were resolved on the multiple forms of some of the enzymes. Several studies concentrated on SV40, a DNA tumor virus, investigating its effect on the synthesis of histones, determining the nucleotide sequence of defective SV40, showing that the genomes of two wild strains of SV40 differed significantly in several areas of the viral DNA.

The Protein Chemistry Section concentrates on chemical and physical aspects of proteins involved in synthetic and differentiation systems of the organisms. Problems currently under study include transfer of isolated chromosomes to mammalian cells, development of granulocytes, fractionation of cells involved in cellular immune reactions, and development of techniques for fractionating proteins.

The Section of Cell Physiology and Oncogenesis emphasized studies of the mechanisms of malignant transformation in cultured cells induced by chemical carcinogens, by viruses or occurring "spontaneously." As previously noted, this section was transferred out of the Laboratory of Biochemistry to DCCP.

Last year the Laboratory of Molecular Biology was expanded by absorbing the resources of the Laboratory of Biology which was deactivated at the time of retirement of its distinguished chief, Dr. Walter Heston. Currently six broad areas are being investigated under the general direction of Dr. I. Pastan:

1. The biochemical basis of malignant transformation with particular emphasis on the role of cyclic AMP and cell surface protein (CSP) in this process. It was observed that the readdition of CSP to many types of transformed cells restored their appearance, surface morphology and alignment to normal by increasing adhesion of these cells to the substratum and to each other. RNA was isolated from normal and RSV-transformed chick embryo fibroblasts. The levels of translatable mRNA for CSP and collagen were diminished in the transformed cells, thus suggesting that the products of the transforming region of RNA tumor viruses act at the gene level to alter cellular behavior.

2. Characterization of the proteins and factors that control cell shape, movement and adhesion. Sufficient quantities of CSP were obtained for physical characterization. Collaborative investigation of the CSP mobility on the surface of cells showed that it moves slower than the intrinsic membrane proteins and does not modulate their mobility.

3. In another project, regulation of gene activity was studied with particular emphasis on the identification of molecules that activate genes and on the determination of the structure and function of the regulatory regions of genes. It was shown that the regulation of the transcription of gal operon is at two overlapping promoters P1 and P2. Cyclic AMP and CRP activate only P1 and inhibit P2, while the gal repressor protein inhibits P1. This allows E. coli to make sufficient galactose metabolites for cell wall biosynthesis when glucose is the carbon source and cyclic AMP levels are low, yet derepress gal enzyme synthesis when galactose becomes the sole carbon source.

4. Studies of the mechanism of site-specific recombination of bacteriophage λ showed that the substrate for integration of λ is a covalent negatively twisted circle, and investigation of the pathway by which single-stranded circular DNAs are converted to double-stranded DNA by E. coli proteins has shown that there are three pathways which share a common

mechanism of DNA elongation but use separate mechanisms of priming the template DNA.

5. Investigation of how the thyroid cell forms, stores and releases its hormone and the elucidation of factors controlling its growth, differentiation and transformation into a malignant cell.

6. Investigations of mammary tumor virus and other oncornaviruses and elucidation of how they are synthesized and how they change normal cells into cancer cells.

Laboratory of Pathophysiology, under the direction of Dr. P. Gullino, continued a broad spectrum of studies on the basic aspects of the development of normal and neoplastic cells.

In the area of mammary tumor pathophysiology, several topics were pursued. Following up on the hypothesis that hormonal deprivation alters the cytosol proteins increasing the availability of the substrate to the lysosomal enzymes of the cells, it was shown that within hours after removal of hormone, the synthesis of cytosol proteins ceased while total protein synthesis continued; hormone removal led to rapid disappearance of microsomal and soluble enzymes of G6PD.

Estrogen receptors were quantitated in a variety of experimental mammary carcinomas. Their translocation into the nuclei during hormone treatment was evaluated, and their reduction in tumors regressing after hormonal deprivation was measured.

In another aspect of mammary tumor pathophysiology it was possible to isolate and purify rat α-lactalbumin (α-LA). Two forms of α-LA were found in rat milk and their amino acid composition has been established. Several mammary tumors produced α-LA and a change in this production was observed with hormonal treatment. In a similar approach, development of the mammary gland was studied in hypothyroid mice, which under standard conditions developed only a rudimentary ductal system. Injection of prolactin, however, induced extensive lobuloalveolar formation. This investigation showed that the epithelial growth as well as the production of α-LA is regulated by the ratio of prolactin to thyroid hormone.

The role of poly(ADP-Ribose) in the initiation of cell division has been further studied. Poly(ADP Ribose) is associated with basic chromosomal proteins, and a major objective was the characterization of this association with particular reference to cell replication. A chromosomal polymerase attaches poly(ADP-Ribose) to H1 histone. The polymer-protein complex is produced by ADP-ribosylation of H1 histone. The poly(ADP-Ribose) chain serves as a bridge linking H1 molecules to form histone dimers. The average chain length of poly(ADP-Ribose) with the complex was found to consist of 15 ADP-Ribose units. The complex is formed by two H1 molecules in association with one molecule of poly(ADP-Ribose). The complex appears to be the same in HeLa cells, human mammary carcinoma cells and normal, lactating and neoplastic mammary tissues of rats. The polymer chain length is controlled

by a poly(ADP-Ribose) glycohydrolase, located in the nuclei. The enzyme has been freed of contaminating phosphodiesterases and nucleases and has been shown to degrade the polymer from the non-reducing end of the chain. It is inhibited by ADPR, cAMP, and single-stranded DNA. Differences between normal and neoplastic mammary tissues in polymer degradation and its role in chromatin protein modifications are being studied.

Under another approach, the behavior of polysomes, ribosomes, ribosomal subunits and messenger RNA related to the onset and cessation of growth in lymphocytes was studied concentrating on the biochemical steps controlling protein synthesis during onset and cessation of cell growth and division. In resting lymphocytes over 70% of the the ribosomes are in a form that does not participate in protein synthesis. After phytohemagglutinin treatment, the increase in protein synthesis depends on the free ribosomes that are considered as a large reservoir ready for utilization. This reservoir is replenished at the peak of cell growth and protein synthesis, as if the cell were anticipating future needs.

Studies of the structure and functions of biological membranes are pursued to gain understanding of cell-to-cell interaction and possibly the mechanism of metastasis.

The study of the pathophysiology of cachexia in the tumor-bearing host continued, as well as the investigation of the role of extranuclear RNA and nucleotide metabolism in normal and neoplastic tissues. The final area currently under active investigation concerns the attempts to understand the mechanisms by which ionizing radiation produces loss of biological activity in DNA and proteins.

The activities of the Laboratory of Theoretical Biology, under the direction of Dr. M. Berman, fall into several broad areas. Most of the work is theoretical, but some experiments are carried out in laboratories also. Much of the theoretical work and modeling is done in collaboration with other experimental groups.

The work on membranes includes a study on the interaction between lipid vesicles and cell membranes. It resembles such physiological processes as secretion and viral infection, and it could be applied to development of vehicles for the delivery of physiologically active agents and drugs to cells or tissues.

The mobility in bimolecular lipid membranes (BLM) of dextrans derivatized with controlled amounts of fatty acid, rhodamine, and TNP has been studied using photobleaching techniques. Diffusion coefficients of these substances were measured, and patching was demonstrated upon addition of anti-TNP or Con A. These studies are part of an effort to elucidate the roles in membrane function played by membrane fluidity and the diffusion of cell surface receptors.

In the area of theoretical immunology, analysis of plaque assay continued with particular emphasis on the experimental conditions which must be fulfilled for inhibition experiments to yield thermodynamic information.

Mathematical models have been developed for describing the dynamic events which occur on the lymphocyte plasma membrane subsequent to interaction with antigen.

Work was also initiated on mathematical modeling of carcinogenesis. The work in this area attempts to formulate a broad conceptual framework within which one can incorporate as much of the phenomenology of carcinogenesis as possible, and to develop mathematical models for specific aspects of carcinogenesis within this framework, that lead to testable predictions or suggestions for original experiments.

The modeling of endocrine and other dynamic systems has continued. In the lipoprotein area, the model developed for the synthesis, catabolism and interconversion of lipoproteins has been confirmed and extended so that it could actually be used for the study of abnormal metabolism. The size of lipoprotein particles plays an important role in the metabolic pathways and can greatly influence the interpretation of kinetic data.

A number of kinetic models have been developed for various systems, including one for the synthesis and metabolism of thyroxine binding globulins and the effects of hyper- and hypothyroidism and of estrogen on these globulins; a model for the synthesis and kinetics of thyroglobulin; and one for the binding of cyclic AMP activation of prostaglandin in normal and transformed cells.

In studying the glucose-insulin control system, an analysis was performed of data in normal, obese, aged and diabetic populations showing relatively comparable kinetics; a reduction in the ability of tissues to retain insulin was noted in cases of diabetes and obesity.

The SAAM computer program developed for the modeling of complex, nonlinear systems has been improved further by extending its power and versatility. Conversational SAAM was extended for a wider use. The staff of the Laboratory of Theoretical Biology also assist many investigators in the use of the SAAM program in a variety of biological problems.

Last year the Laboratory of Immunobiology was transferred from DCCP to the Immunology Program of DCBD. There are now four units under the Associate Director for Immunology: the Immunology Branch and the Laboratories of Immunodiagnosis, Immunobiology and Cell Biology. Although the Metabolism Branch is organizationally under Clinical Research, its progress is summarized here because much of its program content is closer to that of Immunology.

The Immunology Branch (Dr. W. Terry, Chief) is involved in basic studies of the regulation and control of immune responses, structure and function of cell surfaces, transplantation biology and tumor immunology. The research may be captioned under five major subtopics: regulation and control of immune responses, structure and function of cell surfaces, transplantation, tumor immunology and macromolecular biology studies.

An in vitro system was developed for generating antibody responses to soluble antigens in order to study the mechanism of the genetic control of specific antibody synthesis. Results obtained indicate existence of a genetic control of IgM and IgG responses; the macrophage may be the cell involved in regulation.

The in vivo studies of genetic control, using the complex antigen, staphylococcal nuclease, and antibodies against idiotypic determinants produced in rats showed that the idiotype expression is linked to the heavy chain allotype marker, rather than to immune response gene loci.

Functional and serologic characterizations of cells important to the immune response have continued. Studies of the distribution of Ia antigens on various cell populations have shown that spleen macrophages express more I-region antigens than do peritoneal macrophages. Peritoneal adherent cells, usually thought to be mostly macrophages, were shown to contain a small subpopulation of adherent B cells with potent anti-tumor activity.

Many subpopulations of immune and human lymphocytes contain Fc receptors. The Fc receptors of human UL-cells have been shown to have high avidity for the Ig complexes. In addition, it has been shown that Fc receptors of B and T cells are associated with Ia antigen. Fc receptors and Ia antigens are separate molecules on the B cells and their association appears to be ligand induced.

A clinical trial of the use of BCG, and BCG plus immunization with allogeneic cultured melanoma cells has been continued. Data are now being analyzed.

The Laboratory of Immunobiology (Dr. H. Rapp, Chief) is organized in three sections: Cellular Immunity, Humoral Immunity and Immunopathology. In the Cellular Immunity Section attempts were made to delineate the limits of immunotherapy in the treatment of experimental and naturally occurring metastatic cancer. The Humoral Immunity Section studied fundamental mechanisms of complement-mediated cell killing, and the studies in the Immunopathology Section covered the area of tumor-host interactions.

A model was developed in the guinea pigs for early metastases to lymph nodes. Treatment with intralesional BCG eliminated the primary tumor as well as tumor cells in lymph nodes. Various sites of lymph node metastases were established by varying the intradermal site of the challenge inoculum. Various methods of partial and complete cure of this experimental tumor have been experimented with mimicking minimal residual disease in man.

Other studies have been initiated to determine if concomitant immunity to intradermal challenge is detectable during early stages of tumor growth or when a lower initial tumor inoculum is used. The interaction of host "immunity," as manifested by resistance to tumor challenge following radical surgery, with immunoadjuvant administration during advanced stages of malignant disease has been investigated.

In an attempt to find ways for distinguishing specific anti-tumor directed cytotoxicity from "natural killer" activity, a major step was taken by demonstrating that effector cells could be generated efficiently in vitro by culturing lymphocytes with syngeneic tumor cells in the presence of fresh autologous serum. Under those conditions the "natural killer" activity could be eliminated. Effector cells induced by syngeneic hepatoma cells damaged hepatoma target cells but not normal fibroblasts.

In another area, studies were aimed at elucidation of cellular events that make a tumor cell resistant or susceptible to the cytotoxic action of complement (C). Susceptibility of the cells to C killing can be increased by treatment with drugs, metabolic inhibitors, chemotherapeutic agents and with proteolytic enzymes. Treatment of cells with certain hormones led to an increased resistance to killing by C. Increase in the susceptibility of cells to humoral immune attack correlated with complex lipid and not with DNA, RNA and protein synthesis. Treatment of cells with heat, low doses of X-ray or brief exposure to metabolic inhibitors did not increase their susceptibility. Inhibition of synthesis of macromolecules did not correlate with increased susceptibility.

The requirements for hormones to be effective in rendering tumor cells resistant to antibody-C mediated killing were that the cells have at least RNA and protein synthesis intact. The resistance was not due to the physical binding of hormones to the cell. Hormone-treated cells allowed to revert to a susceptible state were not rendered resistant when treated again with the same hormone but were rendered resistant when treated with a different hormone.

Other studies were aimed at elucidation of the nature of the cytotoxic action of the complement and analysis of the mechanism of intralesional tumor treatment by various chemotherapeutic drugs.

In the area of immunologic interactions of host and tumor, macrophages have been reported to be important in host defense against neoplastic disease. Mice that received intraperitoneal injections of BCG developed a chronic infection and tumoricidal macrophages, localized at the site of BCG challenge (peritoneal cavity). Mice injected intradermally did not develop

cytotoxic peritoneal cells, although the mice became immune to BCG antigens. Intraperitoneal injection of soluble BCG antigens (PPD) into these mice induced tumoricidal macrophages. Several functional properties distinguished activated tumoricidal macrophages from normal macrophages or macrophages elicited by irritants. These included increased response to chemotactic stimuli and release of a soluble monokine that was mitogenic for T lymphocytes (LAF). BCG-activated macrophages released 3-5 times more LAF than equal numbers of normal macrophages.

The term lymphotoxin has been applied to the factors in supernatant fluids of stimulated lymphocyte cultures that destroy tumor cell lines, although cell counting assays for lymphotoxin may detect factors that inhibit proliferation as well as those that kill. An assay of cell killing is release of thymidine from prelabeled target cells; it has been used for isolation and purification of mouse lymphotoxin. Further study of the conditions for LT assay has shown that the sensitivity of cells to LT action was greatly increased by tritiated thymidine labeling; this was due to the radiation, not to thymidine.

The research activities of the Laboratory of Immunodiagnosis under the direction of Dr. R. Herberman can be divided into three main areas: cell-mediated immunity studies in animal tumor systems, and in man, and immunochemical studies of tumor-associated antigens (TAA). The main emphasis in each of these areas is on the application of the research information to the diagnosis of cancer and monitoring of the disease.

Lymphoid cells from many normal mice and rats of a variety of inbred strains were found to have reactivity against several syngeneic and allogeneic tumors. There are many similarities in natural killer (NK) cells among various species. Many mouse sarcomas and carcinomas have been found to be susceptible to NK activity. Mouse, rat and human NK cells have considerable reactivity against heterologous target cells; it appears that NK cells can react against a variety of antigenic specificities.

Spontaneous expression of NK reactivity in mice and rats is quite transient; however, it has been possible to augment the levels of NK reactivity in young mice, or to cause a reappearance of NK reactivity in older mice by inoculation of cells bearing the relevant antigen. Recent studies indicate that C. parvum, among other agents, can cause high increases in NK activity in rats. The effector cells mediating the boosted cytotoxicity had the same characteristics as those of NK cells in unstimulated animals.

An increasing amount of data indicates that NK cells may play an important role in in vivo resistance to tumor growth. A number of mouse lymphomas, carcinomas, sarcomas and B16 melanoma have been found to grow poorly in nude mice, which have high levels of NK activity. Each of these tumors has been shown to be sensitive to NK activity. In addition, the fact that some bone marrow cells are sensitive to NK activity, that NK activity

is detectable in the bone marrow, and that inoculation of allogeneic bone marrow cells can boost NK activity is suggestive of a role of NK in bone marrow resistance.

Extensive studies have been performed with the migration inhibition assays, to determine their relationship to in vivo anti-tumor immunity. Spleen cells from animals immunized with MSV, when incubated in culture with antigen to which they had been sensitized, produce migration inhibition factor (MIF). Using populations of immune spleen cells depleted of macrophages by different techniques, it was found that such purified spleen cells were unable to produce MIF when stimulated by soluble antigen. However, the ability to produce MIF could be restored by the addition of normal, syngeneic macrophages which had been previously pulsed with the soluble antigen. Moreover, the macrophage-lymphocyte interaction appeared to be under the control of genes mapping in the MHC (H-2). Finally, it was found that macrophages can interfere with production of MIF in a number of circumstances.

Using the direct agarose microdroplet method, macrophage migration inhibition assays were used to monitor antigenic activity of preparations of mouse SV40 induced tumor cells and of plasmacytomas. The assay was extremely sensitive and could detect migration inhibition reactivity with all three types of antigenic preparations with concentrations as low as 250 picograms protein/ml. The reactivities were quite reproducible and specific for the homologous tumor antigens.

A considerable emphasis has been placed on evaluation of cellular immune competence in cancer patients. This has included the enumeration of the percentage of T cells in the peripheral blood by rosette formation with sheep erythrocytes. In the study of serial monitoring of postoperative lung cancer patients, the rosette results have shown much promise in their clinical correlations, and particularly in their ability to predict recurrence of disease.

Cell-mediated proliferative responses to the mitogens Con A and PHA, and to alloantigens in MLC, were often severely depressed in lung cancer patients, and with lesser frequency in breast cancer. There was some correlation of the depression with the stage of the disease, and a preliminary analysis of the data suggested its usefulness for predicting recurrence.

Delayed hypersensitivity reactions of patients to purified soluble fractions of tumor cell extracts were measured confirming the specificity of this material toward breast cancer. This test is used for serial determinations of all patients in clinical breast cancer protocols to determine the relationship to clinical status and response to therapy.

Leukocyte migration inhibition (LMI) appears to be the most potent in vitro tool to detect TAA on human tumors and tumor-derived cell lines.

Cancer patients with different stages of disease show similar degrees of reactivity, being also positive during relapse. However, the incidence of reactivity appeared to decrease substantially in patients who remained free of disease. A high proportion of breast cancer patients were shown to react in LMI with tissue culture cells derived from breast carcinoma. As in the lung cancer studies, patients who remained free of recurrent disease had substantially lower reactivity than did patients who recurred.

Lung and breast cancer patients were tested for the proliferative responses of their mononuclear peripheral blood leukocytes (MPBL) to autologous tumor material. Sixty percent of the tests in lung carcinoma and 40% in breast carcinoma resulted in clearcut proliferation. Low concentrations of hypotonic membrane preparations worked particularly well in this system. Breast cancer patients usually lose their reactivity after X-irradiation therapy.

The research program of the Laboratory of Cell Biology, directed by Dr. L. Law, is multidirectional, ranging from viral oncology to immunological investigations, mammalian cellular genetics, and chemical and structural studies of antibodies.

Although much work has been done in the areas of tumor biology, the basis of how tissues come to lose their normal cellular control mechanisms and develop the neoplastic state remains to be elucidated. Considerable effort therefore has been concentrated toward a better understanding of neoplastic transformation by adopting as an experimental model system the chemically induced rat hepatoma in tissue culture. During the past year extensive efforts in genome rescue experiments resulted in the isolation of a rat hepatoma virus pseudotype Kirsten sarcoma virus, KiMSV(RHHV), which was then characterized biologically, morphologically, immunologically and biochemically.

In the study of intracisternal A particles (IAP), current efforts are directed toward a comparison of the IAP-associated tRNA methylases with those of the host cell. The results should show whether these methylases are coded for by the particle genome or whether they are simply cell methylases incorporated during the process of replication. It has been established that IAP apparently have a number of tRNA methylases. It has been previously shown that IAP have a number of biochemical markers which are characteristic of RNA tumor viruses. Appropriate experiments indicate that the tRNA methylase specific activity is highest in purified preparations of IAP and that it is bound within the particles rather than simply being associated with the outer surface.

In the study of interferon treatment of oncornavirus infections a differential effect was exhibited by interferon which appeared to be dependent on the cells used to replicate the virus: depending on whether AKR-Ll or SC-1 cells were used, significant inhibition of virus release from the cell was noted or the entire virions remained trapped at the plasma membrane of

the cell. The mechanism of interferon action is associated with membrane phenomena that are critical to a late stage of virus assembly and release. Since interferon could inhibit the translation of a virus protein necessary for assembly, current efforts are directed toward the characterization of interferon-treated virions to determine if there are intrinsic polypeptides missing.

Intensive work has been devoted to the purification and chemical characterization of H-2 antigens. Membranes have been prepared from liver cells by differential centrifugation according to established procedures. The membranes were then digested by limited papain to solubilize H-2 which was purified by DEAE, gel filtration, affinity chromatography on concanavalin, and isoelectric focusing. This material has been shown to be about 80% pure. A single peak of about 40,000 M.W. could be obtained representing the purified heavy chain of the H-2 molecules. A chemical analysis has shown a blocked aminoterminal. Peptide analysis is now in progress.

Studies were continued of the "eclipse" phase of growth of the mKSA neoplasm with the following findings: the host bearing a large tumor develops an "eclipse" phase such that T cells are no longer capable of being sensitized to reject the tumor; immunocompetence returns after surgical removal of the tumor. Other T cell functions examined in an "eclipsed" host appear not to be affected. A factor was identified in the ascitic fluid and in the serum that maintains "eclipse" for a long period of time and which also has growth-promoting activity. Cells which normally will not grow in the syngeneic BALB/c host will grow progressively given the "factor" from ascitic fluid.

In the course of investigations on the immunological response of host animals against reticulum cell neoplasms (RCN) and lymphocytic leukemias, it was discovered that the lymphoid cell suspension prepared from transplantable RCN showed a marked cytotoxic effect against some other tumor cells in culture. This "killer" effect was not indiscriminate, but rather selective, and did not require the presence of macrophages. Only viable cells of well established RCN were able to show this killer activity. The killer cell was found to be Fc positive, Thy 1.2 negative, and not affected by pretreatment with anti-Thy 1.2 and complement. The possibility that the killer activity is exerted by the host cells reacting against RCN is unlikely. It has been postulated that killer cell clones, arising spontaneously and destined for immunological surveillance against some antigens present on transformed cells, were themselves transformed and become neoplastic.

It has been demonstrated previously in both virus-infected mice and in tumor systems that T cell-mediated cytotoxic reactions are H-2 restricted: a strict requirement of compatibility at either the K or D ends of H-2 gene complexes among sensitizing cells, target cells and effector cells. These findings have important implications on tumor immunology: if such strict requirements of histocompatibility genes exist in all tumors, then it will be impossible to prepare a common vaccine for human tumors or find a common tumor antigen for immunodiagnosis. In working with FBL-3 tumor systems, it

was determined that after prolonged incubation time there is no strict requirement of H-2 homology in mediating T cell killing either in the induction phase or effector phase. Furthermore, tumor-associated antigens are entirely different from H-2 antigens, i.e. only tumor antigen can inhibit cytotoxic reactions, not H-2 antigen, and only anti-tumor antibodies can suppress the induction of cytotoxic response to tumor-associated antigens, not anti H-2 antibodies.

In the investigation of mammalian cell genetics, the investigators of this laboratory have produced and characterized several valuable chloramphenicol-resistant (CAP-r) mutants and fingerprinted their mitochondrial DNA (mt-DNA). They repeated and extended the findings that CAP-r mt-DNA could "transform" chloramphenicol-sensitive cells (CAP-s) to CAP-r, showing that three subspecies of Mus could be used to confer the CAP-r phenotype among one another both by cytoplast fusion and by treatment with CAP-r mt-DNA; they acquired new techniques for nick translation of nanogram quantities of mt-DNA using ^{32}P-nucleotides so that small quantities of restriction fragments might be visualized by autoradiography and for labeling with kinase so that the smaller fragments might be seen.

Considerable effort was devoted to chemical and structural studies of antibodies. A detailed analysis was carried out on the heavy chain of the immunoglobulin secreted by mouse myeloma MOPC 47A, determining the complete amino acid sequence. Investigation of rabbit immunoglobulins, specifically of the ligand-binding role of hypervariable region residues, showed that light chain tyrosyl 30 is involved in such ligand binding. Complete heavy chain variable region sequences have been obtained for levan-binding proteins; a three-dimensional model was constructed for anti-levan protein, and finally galactan-binding protein was obtained in crystalline state and three-dimensional analysis of this protein has begun.

The research program of the Metabolism Branch, under the direction of Dr. T. Waldmann, is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area investigations are carried out to determine the nature of immune processes that are of importance in the surveillance against neoplastic cells and to define the nature of defects in the immunological system that result in an increased incidence of neoplasia. The second major goal is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Within this area special emphasis is laid on the biochemical events accompanying normal growth and the hormonal control of this growth as it relates to our understanding of malignant growth.

A major effort has been directed toward defining the events of cellular differentiation, interaction and biosynthesis involved in the specific antibody response. Special emphasis was placed on defining the defects of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with primary immunodeficiency diseases, autoimmune disorders and malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states

controlling the production of antibodies and the synthesis of immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then into immunoglobulin-synthesizing plasma cells. The maturation of B cells is regulated by a series of other cells. Many antigens require both helper lymphocytes of thymic origin and macrophages as well as the B cells to induce a full antibody response. Over the past year a series of studies were initiated to determine whether homogenous populations of T cells from patients with T cell leukemias retain the capacity to help or suppress the synthesis of immunoglobulins from lymphocytes derived from normal individuals. The initial studies focused on patients with the Sezary syndrome -- a malignant disease characterized by exfoliative erythroderma, generalized lymphadenopathy and circulating neoplastic T cells. Lymphocytes from patients with the Sezary syndrome synthesized essentially no immunoglobulin in pokeweed mitogen-stimulated cultures in vitro; they possessed no suppressor capacity. However, the circulating lymphocytes from approximately 70% of the patients functioned as helper T cells. There was an augmentation of immunoglobulin production by purified normal B cells after the addition of Sezary cells. These studies support the view that the Sezary syndrome represents a homogenous malignant expansion of helper T cells.

As part of the studies in man, a system of autoreactivity present in normal animals was investigated. An example of autoreactivity is the capacity of T lymphocytes to respond to B lymphocytes from the same individual. In vitro, this response is demonstrated with autologous B lymphocytes. A variety of substances with immunoregulatory properties were studied for their effects on this form of lymphocyte autoreactivity. At physiological and supraphysiological concentrations of alpha-fetoprotein and the thymic hormone, thymosin, no detectable effect was observed on the autologous MLR. Cortisol at pharmacologic concentrations has been shown to be suppressive to a variety of in vivo and in vitro lymphocyte functions. Thus, concentrations of cortisol found normally in human plasma have a profound immunoregulatory role in selectively blocking autoreactivity of at least one class of immunocompetent T cells.

Discovery of the existence of immune response (Ir) genes, which control the ability of an animal to respond to an antigen, has given us an opportunity to understand the regulatory factors of immune surveillance against malignancy and infectious disease as well as the abnormal immune responses involved in the pathogenesis of autoimmune diseases. A study in this laboratory has shown that the control by H-2 genes can be exerted at the level of individual antigenic determinants rather than the whole antigen molecule. It has been shown that an additional gene linked to neither H-2 nor heavy chain immunoglobulin allotype controls the overall level of response to the whole molecule which can be reached after multiple immunizations, while the H-2 linked genes control the proportions of antibodies made to different determinants.

Gluten-sensitive enteropathy (GSE) is a disease of the GI tract associated with malabsorption, villous flattening, inflammatory cell infiltration of the GI tract and an increased incidence of neoplasia. It is due to the

toxic effect of wheat proteins on the gastrointestinal mucosa. Approximately 80% of patients with GSE bear the HLA-B8 histocompatibility antigen, in contrast to 35% of random control individuals. In a prospective study of patients with diarrheal illness, tissue obtained from 23 GSE patients were subjected to organ culture in the presence and absence of gluten protein. Nineteen of them demonstrated gluten toxicity in organ culture; all of these patients were HLA-B8 positive. These studies point to the conclusion that "HLA-B8 status" in some measure determines the degree of toxicity to gluten protein. Analysis of the inheritance of GSE in families studied in relation to the inheritance of HLA antigens showed that HLA antigens cannot be the only genetic determinants important to the occurrence of GSE. A second genetically determined cell surface protein associated with GSE was discovered -- the GSE-associated B cell antigen. Using a cytotoxicity test specific for B cells it was determined that the B cell antigen is present in 90% of patients, but only 20% of normals.

Using the typing methods for HLA-DW3 and GSE-associated B cell antigens, individuals in seven families of patients with GSE were characterized. It was found that all parents of patients were positive for the B cell antigen; the high degree of positivity among parents strongly suggests that patients were homozygous for the GSE-associated B cell antigen. Most patients were positive for HLA-DW3; it is therefore likely that at least a single dose of the gene controlling HLA-DW3 is necessary for the occurrence of GSE. The inheritance of the GSE-associated B cell antigen and HLA antigen was discordant: in several families siblings were found who were GSE-associated B cell antigen identical, but HLA non-identical. These studies indicate that GSE is controlled by two non-linked genes, one in the major histocompatibility locus.

The studies using sensitive radioimmunoassays for the oncofetal proteins, alpha-fetoprotein (AFP) and human chorionic gonadotropin (hCG), have continued. A new radioimmunoassay for alpha-fetoprotein has been established that significantly extends the lower limit of detectability of this protein. There are virtually no elevations of alpha-fetoprotein in benign conditions and physiological states other than pregnancy. AFP is elevated in the ataxia telangiectasia and disorders associated with extreme hepatocellular regeneration: 79 of 80 patients with ataxia telangiectasia had an elevated alpha-fetoprotein level. The combined markers alpha-fetoprotein and human chorionic gonadotropin are of great value for following the therapy of hepatocellular tumors, and germ cell tumors of the testis other than seminomas. There have been no false positive values utilizing these markers and only 10% false negative values. Alpha-fetoprotein and human chorionic gonadotropin are produced by separate cells in the testicular tumors and one may have a discordance of these markers during chemotherapy when there is a difference in the response of the different tumor elements to therapy. Thus, both markers should be measured at the time of diagnosis during patient therapy.

A radioimmunoassay for multiplication-stimulating activity (MSA), a family of polypeptides, has recently been developed and studies are under way to study the physiology of MSA in the rat, including site of synthesis and circulating levels.

CLINICAL RESEARCH

The report of Clinical Research includes work in the Dermatology Branch and the Laboratory of Pathology. The Diagnosis Program is a collaborative, extramural effort and will not be reported here. The clinical programs of the Metabolism Branch were described in the preceding section with the Immunology Program.

The Dermatology Branch (Dr. M. Lutzner, Chief) conducts both clinical and laboratory research on the etiology, diagnosis and treatment of diseases involving the skin and host's response to these diseases. The Branch also provides dermatologic consultation to all other services of the Clinical Center (approximately 800 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year follow.

Exploratory studies of a new class of vitamin A derivatives were initiated in the treatment of skin cancer and diseases of keratinization. Patients with treatment-resistant disorders of keratinization were treated with oral 13-cis retinoic acid; responses varied from excellent to none, depending on the specific disease. Excellent results were seen in patients with congenital ichthyosiform erythroderma, lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris and cystic acne. Commonly observed side effects included cheilitis, facial dermatitis, xerosis, skin fragility, and conjunctivitis. The mechanism by which this synthetic retinoid alters these disease states is not clear but may be related to the observed ability of vitamin A to affect glycoprotein synthesis and epithelial differentiation.

The epidermis offers a unique opportunity for the study of tumors because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis could lead to the discovery of such a marker. The seven polypeptide chains which comprise the subunits of the keratin filaments of normal bovine epidermis have been isolated and individually characterized by standard protein chemical techniques. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis with those of normal epidermis show prominent differences in numbers and mobilities of bands.

Ultraviolet radiation is believed to be a major cause of the most common type of human cancer, cancer of the skin. Patients with xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. XP patients' sun-exposed skin ages much more rapidly than normal human skin. Some XP patients develop neurological

abnormalities which are due to the early death of neurons. The studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and these aging phenomena in XP.

Warts represent a viral-induced tumor of man. A closely related virus, the Shope papilloma virus of rabbits, will induce benign tumors in some rabbits and malignant tumor in others. The squamous cell cancers of patients with epidermodysplasia verruciformis may be induced by wart viruses. Methods are being developed which should not only permit the screening of tumors for wart viral material, but also facilitate attempts to propagate the virus in tissue culture.

Studies of the immunopathologic mechanisms involved in inflammatory skin diseases have emphasized pemphigus, herpes gestationis, bullous pemphigoid, and dermatitis herpetiformis. Serum and in vivo bound antibodies in these diseases were identified and characterized. The efforts have centered around the mechanisms of tissue destruction, with ultrastructural localization of antibodies.

The abnormal proteins produced in malignant melanoma are being studied: these studies are aimed at elucidating the mechanism of formation of these proteins, as well as their role in the immunology of melanoma. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules resolved a slight, but significant, difference of isoelectric points. The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by neoplastic transformation. The results indicate that although melanogenesis takes place in the malignant cells its metabolic pattern is abnormal.

The diagnosis and research activities of the Laboratory of Pathology (Dr. L. Thomas, Chief) are oriented in a number of directions, reflecting sectional organization: hematopathology and immunopathology, viral oncology and cell biology, comparative pathology and carcinogenesis, automation of equipment for cytology diagnoses and clinicopathologic studies on a variety of human diseases. In addition to research being carried out, the staff participates in diagnostic services for Clinical Center patients.

Some of the significant research and clinicopathologic accomplishments in which the staff of the Laboratory participated, either as principal investigators or as collaborators, include:

Further characterization of the morphology, immunoglobulin production and surface membrane characteristics of cells of human lymphomas, particularly the cells of "hairy" cell leukemia, and the presence of distinctive lesions, pseudosinusoids in the spleen and angiomatous structures in the liver, associated with "hairy" cell leukemia.

Pathologic and statistical studies of reviewed breast cancer cases from the HIP Breast Cancer Study and the relationship of these findings to the assessment of risks and benefits of including mammography in the routine screening of asymptomatic populations of women for breast cancer.

Development of experimental and mathematical models for the study of metastases in animals. Demonstration that access of fibrosarcoma cells to the circulation and the number of metastases may be suppressed by BCG-stimulated peritoneal macrophages inoculated intravenously.

Approximately 7,140 surgical specimens and 244 autopsies were examined in the past year by the surgical pathology and postmortem service. These specimens involved in the preparation of more than 61,000 slides including nearly 44,000 H & E stained sections, more than 7,500 special stains and 468 frozen sections.

The Cytopathology Section provided diagnostic service in exfoliative cytology, medical cytogenetics and fine needle aspiration. Chromosomal analysis includes standard karyotyping and special techniques for the identification of individual chromosome pairs. During the year more than 4,000 cytology specimens were accessioned. These represented over 9,500 smears which were examined and reported. Approximately 1/3 of these were vaginal and cervical specimens and nearly 2/3 were other types of specimens including ascitic, pleural, gastric, prostatic and spinal fluids, bronchial specimens, wound washings and fine needle aspirations.

Members of the Quantitative Cytology Section are responsible both for the intramural research and for supervision of the extramural contracts under the DCBD Cytology Automation Program. The intramural research includes several major projects. Among them is the use of cytochemical methods for analyzing the macromolecular content of cells such as use of propidium iodide-fluorescein isothiocyanate staining of gynecologic specimens and analysis of the histone content and histone fractions of cells.

A second major effort is to develop analytic techniques for multiparameter flow data. The LASL sorter is now on line with the PDP 11/40 system on the fifth floor, and work is in progress with taped data to evaluate a number of algorithms for quantitative definition of normal and abnormal gynecologic cytologic materials stained with PI-FITC.

Two projects, one involving analysis and sorting of chromosomes and the other involving analysis of both DNA content and DNA synthetic rate in individual cells, were extensively explored with the LASL sorter. These experiments were then terminated, the former because of marginal sensitivity of the instrument and the latter because of commercial availability of better equipment and methodology.

The Image Processing Unit (IPU) was transferred to the Quantitative Cytology Section in March 1977. The staff of IPU have developed computer systems for on-line measurement and characterization of both fixed and living cells. Extensive optical and image processing equipment and computer hardware have been constructed together with the development of appropriate software systems. The IPU will continue working in computer science but will direct their major research efforts to biological problems. These efforts will also involve scientific collaboration and supplementation between the ongoing intramural research of the Quantitative Cytology

Section using the LASL sorters and the IPU using the image processing techniques and equipment which they have developed.

The members of the Comparative Oncology Section continued the work on epithelial-mesenchymal relationships in neoplasia. During the past year it was demonstrated that neoplastic transformation of polyoma virus-infected epithelial cells cultured on Millipore filters can be induced in the absence of mesenchyme. Four transformed lines are in continuous culture and produce tumors when transplanted into syngeneic mice. A major finding from these studies is that the tumor lines are different from those induced in the same cell type in the presence of salivary gland mesenchyme in that they are more highly invasive and frequently metastasize.

Studies were continued on the pulmonary carcinogenesis with polynuclear hydrocarbons and tobacco products. In these experiments the influence of various local factors including nitrates, vitamin A analogs and polynuclear inhibitors on tobacco-induced pulmonary cancers is being examined. Studies on the tumorigenicity of various fibrous materials and fiber sizes are being continued. These include fibrous glass, potassium titanate, aluminum oxide, dawsonite, silicon carbide, gypsum and talc. Large-diameter fibers and minute short fibers are adequately handled by phagocytic activity, the latter by phagocytosis, the former by a process of fusion and plating of the surface of the particulate material. However, fibers that have diameters <1.5 μm and lengths greater than 8 μm are not handled by phagocytes but initiate collagen deposition and result in a high incidence of pleural neoplasms.

The staff of the Pathological Technology Section provide histological sections and special stains for animal tissues for scientists in the DCBD and other NCI Divisions. During the year they processed more than 31,000 blocks of tissue, prepared over 41,000 H & E slides and more than 3,000 special stains. There were a total of 108 NCI investigators who submitted material for sectioning. Also during the year 154 blocks of tissue were embedded for E.M. Of these, sections were prepared on approximately 140. Two hundred eight photomicrographs were taken and nearly 500 pictures were printed. The quality and performance of this section's staff continue to be outstanding and they represent a major technical resource for the research of scientists in the NCI.

CONTRACT RESEARCH PROGRAM

The Division of Cancer Biology and Diagnosis supports three separate program thrusts through contract mechanism: Tumor Immunology Program, Breast Cancer Task Force Program and Cancer Diagnosis Program.

The largest effort is in the area of Tumor Immunology, managed by the Associate Director for Immunology. The program consists of four operational components: Immunobiology, Immunodiagnosis, Immunology of Cancer Cause and Prevention and Immunotherapy. The goals of the program are (1) the use of immunologic techniques to detect agents etiologic for cancers, (2) the prevention of cancer by immunologic manipulations, (3) the use of immunologic techniques to improve the diagnosis of cancer, and (4) the manipulation of the immune system of patients with established cancer so that increased survival is achieved.

Immunobiology program supports research aimed at acquiring understanding of the nature of cell surfaces and the interaction of the immune system with tumor cells.

Cellular and humoral immune responses to tumors have been investigated and the role of lymphocyte subpopulations and macrophages in immunity against tumors has been analyzed. Studies are under way on circulating immunosuppressive factors and the role of suppressor cells in tumor responses was investigated. In the area of immunodiagnosis, the goal is to develop and evaluate tests which could be useful in detection and diagnosis of cancer and in prognosis of the course of the disease. The research concentrates on circulating tumor antigens and on immune responses to tumor antigens.

The goals of the Immunology of Cancer Cause and Prevention Program are to develop immunologic methods that may be useful in experimental studies on cancer cause and prevention. Means are being sought to detect carcinogens in the environment and in body fluids and organs, to uncover and correct possible immunologic defects accompanying carcinogenesis and to inhibit tumorigenesis before the development of recognizable malignancy.

Immunotherapy program aims to identify immunologically active agents that lead to cure, regression or stabilization of established tumors; to determine the mechanisms whereby such agents work; and to assess the effectiveness of these agents in patients with cancer.

Breast Cancer Task Force program formally covers four broad areas of investigation: Diagnosis, Epidemiology, Experimental Biology and Therapy. Specifically, the research thrusts include work on genetic and environmental factors of breast cancer, predictive indices for the behavior of breast cancer, attempts to isolate, cultivate and characterize normal and neoplastic mammary tissue, hormonal dependence and response to hormonal manipulations, detection and diagnosis and host-tumor relationships affecting the defenses

of the host and growth potential of mammary neoplasia. A series of contracts support the program with necessary resources.

Broadly speaking, the Cancer Diagnosis Program extends into five areas: General Diagnosis, Radiology, Immunodiagnosis, Breast Cancer Diagnosis and Cytology Automation. The first two comprise a separate organizational unit and are reported here. The others are discussed separately.

In the general diagnosis area the program includes three screening studies (cancer of the lung, colon and pancreas), and evaluation of multisite screening models as well as of the periodic screening for medullary thyroid carcinoma. The search for tumor markers continues and the study of the application of NMR to cancer diagnosis has been brought to completion. In the Diagnostic Radiology program considerable effort has been devoted to computerized axial tomography, and on development of various algorithms for this modality. New tissue imaging sources were sought through the projects on proton beam and heavy ion beam. Due to the continuing appearance of new imaging techniques, standard protocol for their evaluation is being developed and will be pilot-tested in the near future.

The Cytology Automation Program covers six areas of interest: specimen collection and preparation, development and evaluation of integrated screening systems, identification of quantitative descriptors of pre-malignant and malignant lesions and development of algorithms for classification of single cells. Finally, work has been initiated on comparison of instrumental classification with the "true" microscopic classification utilizing standard morphological criteria.

LABORATORY OF BIOCHEMISTRY

I. INTRODUCTION

II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Head)

- A. Use of Somatic Cell Genetics to Study Steroid Responsiveness.
- B. Characterization of Glucocorticoid Receptor (GR).
- C. Studies on Gene Control Using Enucleated Cells and "Cybrids".
- D. Clinically Related Studies.
- E. Cell-free Translation.
- F. Miscellaneous.

III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Head)

- A. Collagen Synthesis.
- B. DNA Polymerases.
- C. Cellular Interactions in the Immune Response.
- D. Structures of Macromolecules.
- E. Intracisternal Type-A Particles.
- F. Transcription of Host-Substituted SV40 DNA.

IV. CELL PHYSIOLOGY AND ONCOGENESIS SECTION (Dr. K. Sanford, Head)

- A. Visible Light and Oxygen Effects on DNA, Chromosomes and Transformation Cells in Vitro.
- B. Fluorescent Light Effects on Proliferation and Lifespan of Human Cells in Culture.
- C. Methylation of Eukaryotic DNA.
- D. Methylases in the Intracysternal A Particles.
- E. Role of Oxygen for Survival, Growth, and Metabolism of Sparse Cell Populations.
- F. Analyses of a Peptide-Like Growth Factor for Mammalian Cells in Serum-Free Medium.
- G. Procurement and Cultivation of Human Epithelial Cells.
- H. Morphology of Transformation in Human and Rodent Cells.
- I. The Topography of Cells in Culture.
- J. Locomotor Behavior of Nonneoplastic and Neoplastic Fibroblasts in Culture.

V. CELLULAR REGULATION SECTION (Dr. R. F. Goldberger, Head)

- A. Purification and Translation of Vitellogenin mRNA and Synthesis of cDNA.
- B. Development of in Vitro Systems to Study Transcription of the Vitellogenin Gene.
- C. Organization of Estrogen Responsive Genes in the Avian Genome.

VI. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. M. F. Singer, Head)

- A. Polynucleotide Phosphorylase and Cyclic AMP Phosphodiesterase.
- B. Binding of H1 Histone to DNA.
- C. Effects of H3 and H4 Histones on the Physical State of DNA.
- D. Effect of SV40 Infection on Histone Synthesis.

E. Genomic Differences Between Two Wild Type SV40 Strains.
F. Sequence Analysis of Defective SV40 DNA and Highly Repetitive Monkey DNA.

VII. PROTEIN CHEMISTRY SECTION (Dr. E. Peterson, Head)

A. Chromosome Transfer.
B. Granulocyte Development.
C. Cellular Immune Reactions: Cell Separations.
D. Displacement Chromatography of Proteins.

VIII. INDIVIDUAL PROJECT REPORTS

SUMMARY

Annual Report of the LABORATORY OF BIOCHEMISTRY, National Cancer Institute
July 1, 1976 through September 30, 1977

I. INTRODUCTION

This year in the Laboratory of Biochemistry (Dr. R. Goldberger, Chief) there was a good deal of scientific progress, a general feeling of enthusiasm, and a major reorganization. The Cell Physiology and Oncogenesis Section has recently moved to the Division of Cancer Cause and Prevention, and a new section will take its place -- the Developmental Biology Section. This Section will be headed by Dr. Igor Dawid, who will move from the Carnegie Institution early in 1978. In addition, there has been an influx of outstanding young scientists. Major changes in space have allowed us to construct a P3 facility for pursuing the experiments in molecular cloning that have been planned for some time, an electron microscopy suite that will at last meet the needs of the many scientists now interested in molecular microscopy, and an enlarged and versatile dark room.

The Laboratory of Biochemistry is divided into six Sections. The Biochemistry of Gene Expression Section studies control of gene expression in eukaryotic cells and its application to clinical medicine. The effects of steroid hormones on enzyme induction and cell function in tissue culture are investigated, along with steroid receptors in cells from patients with leukemia and breast cancer. The Biosynthesis Section is concerned with the relationship between cell function and organization at the molecular level. Biochemical, tissue culture, cell fractionation, and electron microscope techniques are employed. In the Cell Physiology and Oncogenesis Section the major objective is to elucidate the mechanisms of so-called "spontaneous", virus-induced, and chemical carcinogen-induced malignant transformation in cultured cells. Research toward this objective includes studies on cell growth and nutrition, cytology, ultrastructure, immunology and biochemistry. The Cellular Regulation Section studies regulation of gene expression in eukaryotes at the molecular level. The central problem under investigation is the mechanism by which estrogen induces the synthesis of egg yolk phosphoprotein in rooster liver, with special emphasis on the molecules involved in the interactions of hormone, hormone receptor, RNA polymerase and chromatin that result in the synthesis of

transcripts of the phosphoprotein gene. The Nucleic Acid Enzymology Section is concerned with detection and study of enzymes involved in nucleic acid synthesis and degradation. Systems under investigation include DNA enzymes related to simian virus 40 replication, structure of host-substituted simian virus 40 DNA, interaction of histones with DNA, bacterial restriction endonuclease, and bacterial polynucleotide phosphorylase and cyclic AMP phosphodiesterase. The Protein Chemistry Section emphasizes the physical, chemical, and biochemical aspects of proteins and subcellular particulates involved in the synthetic, control, and differentiation systems of organisms. Problems currently under study include transfer of isolated chromosomes to mammalian cells, granulocyte development, fractionation of cells involved in cellular immune reactions, and techniques for fractionation of proteins.

II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Head)

A. Use of Somatic Cell Genetics to Study Steroid Responsiveness. Preliminary results suggest that the inhibitory effects of corticosteroids on L cells are recessive in hybrids between these cells and the resistant hepatoma cell line, HTC. The CEM line of human leukemic cells has been characterized with respect to several steroid responses by Dr. Norman. These cells lyse in the presence of steroids. Resistant clones, appearing from sensitive clonal populations at a frequency of about 1×10^{-6}, have been isolated. Killing curves with a variety of mutagens are being carried out, as is fluctuation analysis, by Dr. Harmon. It is planned to isolate a series of resistant clones, characterize them as steroid receptor mutants or as extra-receptor mutants, and analyze them by cell hybridization with appropriate parents to test for dominance/recessiveness, complementation, and mapping of certain steroid-related functions.

B. Characterization of Glucocorticoid Receptor (GR). At an early stage of purification, it became clear that GR does not have properties analogous to those of the progesterone receptor (PR). Dr. Sakaue can distinguish two forms of GR by DEAE-cellulose chromatography. Their properties differ from those described for the two non-identical subunits of PR (which run similarly on DEAE), and preliminary experiments suggest that one may be the "preactivated" and the other the "activated" steroid-receptor complex.

C. Studies on Gene Control Using Enucleated Cells and "Cybrids". 1) The studies reported last year on "cybrids" between enucleated L cells and mouse erythroleukemic (MEL) cells have been extended to neuroblastoma x MEL cell cybrids. Inducibility of hemoglobin is lost or reduced in the cybrids. 2) The reversible, rapid inactivation of tyrosine aminotransferase (TAT) in HTC cells by concanavalin A has been explored in detail. 3) By attaching them to conA-treated coverslips, HTC cells have been enucleated, and the TAT remaining in the cytoplast has been found to be stabilized due to reduced protein degradation.

D. Clinically Related Studies. Biopsies from about 650 patients with breast cancer have been examined for steroid receptors. Among these patients have been a few males. A case report on the first of these, showing estrogen receptors in the tumor has been published. A prospective treatment protocol is underway. Cells from five patients with Sezary's syndrome and from mis-

cellaneous other blood dyscrasias have been analyzed for receptor content by Dr. Schmidt. The rare Sezary cells are of special interest because they have been shown to be of "helper T-cell" origin; thus, studies of steroid effects may pertain to the pharmacology of the hormones on immunologic functions as well as to antileukemic effects. The estrogenization which sometimes follows use of the antidiuretic steroid, spironolactone, may be due to the drug's occupancy of androgen receptor sites. It has been shown that the drug competes for such sites in rat ventral prostate.

E. Cell-free Translation of mRNAs for tyrosine aminotransferase and prolactin is being developed by Drs. Schmidt, Thompson and Paterson, and Mr. Buckler to try to estimate functional levels of the corresponding messenger RNAs during induction by steroids or other hormones in appropriate cell systems.

F. Miscellaneous. 1) Mutagenesis in xeroderma pigmentosum cells has been studied by Drs. Lyons and Robins (C/D), using development of ouabain resistance following UV light; survival of irradiated cells in vitro has been correlated with the clinical type of xeroderma pigmentosum from which the cells were obtained. 2) The ontogeny of glucocorticoid receptors in early mouse embryos showing high and low teratogenesis after glucocorticoids has been studied by Drs. David Salomen (D/LDBA), Zubairi, and Thompson. 3) Experiments suggesting the existence of receptors for oxygenated sterols have been successful (Drs. A. Kandutsch and H. L. Chen, The Jackson Laboratories). 4) During myogenesis in chick muscle, the sequence complexity, abundance classes and coding capacities of mRNAs have been characterized by Dr. Paterson in collaboration with Dr. John Bishop (University of Edinburgh). 5) Other collaborations include NMR analysis of normal vs transformed cell lines (Drs. R. Ader and J. Cohen, A/LCP); investigations of effect of corticosteroids on C-type virus induction (Dr.Zubairi); studies of the role of the 5'-methyl-G "cap" in mRNA translation; the arrangement of AMV structural genes through tryptic peptide analysis of cell-free AMV RNA translation products; and translation of A particle RNA in vitro.

III. BIOSYNTHESIS SECTION (Dr. E.L. Kuff, Head)

A. Collagen Synthesis. Dr. Peterkofsky and her associates have continued to study the reducing factors for proline hydroxylation in cultured mouse cells. Hydroxylation is highly dependent upon ascorbate in non-transformed BALB/c 3T3 fibroblasts. However, Kirsten sarcoma virus-transformed cells (Ki-3T3) show 60 to 90% of the maximum proline hydroxylation in the absence of ascorbate. L-929 fibroblasts also exhibit ascorbate-independent hydroxylation, but only in the stationary growth phase. An assay was developed which permits measurement of reducing cofactor activity in cell extracts; it was shown that ascorbate-independent proline hydroxylation is due to a reducing substance other than ascorbate that is present in greater amounts in Ki-3T3 cells than in 3T3 cells, and in greater amounts in stationary cells than in log phase L-929 cells. The cofactor can exist in oxidized (inactive) and reduced (active) states, the relative proportions of which appear to be regulated by the incracellular level of NADH. The cofactor is not tetrahydropterin or tetrahydrofolate. In another study, Dr. Peterkofsky and coworkers have compared the collagen phenotypes of contact-inhibited mouse fibroblasts (3T3) and

of cells transformed by murine sarcoma viruses, SV40, and a chemical carcinogen respectively. The results suggest that the collagen phenotype - that is, the specific collagen genes expressed by the cell - can be altered by transformation and that the changes may be specific for certain transforming agents.

B. DNA Polymerases. As part of a continuing study of the enzymatic mechanisms of DNA synthesis, Dr. Wilson and his colleagues have developed an improved method for the rapid purification of DNA polymerase alpha from mouse myeloma. Salient new features include measures to reduce cell protease activities and the use of oligo(dT)-cellulose chromatography. The alpha polymerase is obtained in nearly homogenous form, with a recovery of approximately 20%. SDS-polyacrylamide gel electrophoresis reveals components with apparent molecular weights of 235,000, 176,000 and 51,000. The largest protein is thought to represent the intact enzyme. This polypeptide may be very sensitive to a site-specific cleavage which gives rise to the two smaller chains. Another aspect of this program is the characterization of protein factors that can modify the activity of the isolated polymerases. In this regard, a group of single-strand DNA-binding proteins has been isolated from myeloma cells by chromatography on DNA-cellulose and further resolved by isoelectric focussing in polyacrylamide gels. They represent a mixture of 10 to 15 polypeptides comprising about 1% of the total cell protein. The individual proteins are being characterized with respect to DNA unwinding ability and effects on enzymatic activity. In addition to these typical DNA-binding proteins, a protein factor has been identified that greatly increases the capacity of the alpha-polymerase to replicate single stranded calf thymus DNA. The factor is completely free of DNA polymerase activity, is active at a very low protein to template ratio, and may act through facilitating elongation of nascent chains.

C. Cellular Interactions in the Immune Reponse. Dr. Shelton has extended her studies on the effect of various inhibitors and of temperature on the surface topography of mouse lymphocytes, macrophages and mast cells. The mast cell surface proved very resistant to the effects of these agents. Inhibitors of energy metabolism are promoters of microvillus extension on lymphocytes and macrophages. Local anaesthetics and diamide (a glutathione-oxidizing agent) are inhibitors of microvillus extension. Low temperature affects surface topography and over-rides the effects of anti-metabolites. Low temperature and diamide compete; in the presence of both, the cell surface appears normal. These promoters and inhibitors provide a way to examine the regulation of cell surface topography.

D. Structures of Macromolecules. In collaboration with Drs. P. Plotz and R. Guyer (A & R, NIAMDD) and Drs. H. Gewurz and A. Osmand (Rush Medical College), Dr. Shelton has examined the ultrastructure of C-reactive protein (CRP), a component of serum whose concentration increases dramatically in inflammation. The molecule has been shown to consist of 5 protomers arranged in cyclic symmetry at the vertices of a regular pentagon. Both human and rabbit CRP measure 11 nM in width and the individual protomers are 3.8 nM in diameter. A molecular weight of 23,700 was calculated, in good agreement with the value of 22,900 found by sedimentation analysis. Pentameric molecules are so rare that a new class called pentraxins has been proposed.

E. Intracisternal Type-A Particles. Drs. Lueders and Kuff continued studies with particular emphasis on defining the genetic information associated with virus-like type-A particles. Using cDNA prepared from the polyadenylated RNA of mouse myeloma A-particles, they identified a group of RNA sequences that were much more abundant in cells producing A-particles than in particle-negative cells and were concentrated in A-particles isolated from three very different tumor cell types. This group of sequences had a genetic complexity (estimated from kinetic data) of at least 5000 nucleotides. The sequences were reiterated between 2000 and 4000 times in the genome of normal mouse cells (liver and sperm). The copy number was not higher in cells producing A-particles. In situ hybridization of mouse chromosomes with ^{3}H-cDNA indicated that these sequences had a widespread non-centromeric distribution as well as being concentrated in definite regions of certain chromosomes. With Dr. B. Paterson (Biochemistry of Gene Expression Section), it was shown that poly(A)-RNA from myeloma A-particles directed the in vitro synthesis of a 73,000 dalton product that was identified as the main A-particle structural protein on the basis of its methionine-containing tryptic peptides.

F. Transcription of Host-Substituted SV40 DNA. Dr. Kuff, in collaboration with Dr. G. Khoury (LDNATV, NCI), has been studying the transcription of a defective SV40 variant in which parts of the closed circular viral DNA have been deleted and replaced with covalently linked host cell DNA. They have examined the occurrence of SV40 and host sequences among the RNA synthesized by intact infected cells and by extracted viral transcriptional complexes incubated in an in vitro system. The results indicate that the substituted host sequences are transcribed but that the transcripts are preferentially degraded in the intact cells. The findings are relevant to the transcription of laboratory-prepared recombinants vectored in SV40 and to the more general problem of RNA processing in eukaryotic cells.

IV. CELL PHYSIOLOGY AND ONCOGENESIS SECTION (Dr. K. K. Sanford, Head)

A. Visible Light and Oxygen Effects on DNA Chromosomes and Transformation Cells in Vitro (Drs. Sanford, Gantt and coworkers in collaboration with Dr. Ram Parshad, Howard University School of Medicine). The DNA of mouse cell cultures exposed to fluorescent light (150 foot-candles, 20 hours) eluted more slowly in the Kohn alkaline elution assay than did that from the shielded controls. This slower elution is interpreted to mean that the DNA is crosslinked in some way with protein and/or DNA. Visible light and oxygen were also found to cause chromatin breaks. Experiments indicate that the effective wavelength for both DNA crosslinking and chromatin breaks lies between 400 and 450 nm. The addition of catalase or glutathione to the culture medium during light exposure significantly reduces or abolishes the incidence of light-induced chromosome aberrations. In limited studies to date, repeated exposure of mouse cells to fluorescent light produces or accelerates neoplastic transformation as compared with cultures shielded from light during routine handling. These results suggest that production of H_2O_2 and a high redox potential of the medium are causative factors in the karyotypic instability of mouse cells and that karyotypic instability and/or DNA damage produced by singlet oxygen may be a causative factor in neoplastic transformation of rodent cells in culture.

B. Fluorescent Light Effects on Proliferation and Lifespan of Human Cells in Culture (Dr. Sanford in collaboration with Dr. R. Parshad, Howard University School of Medicine). Exposure of human diploid fibroblasts to fluorescent light (3-4 hours, 1-3 X weekly) reproducibly enhances the proliferation rate and extends the lifespan of human diploid fibroblasts in culture. Single or multiple exposures of short duration stimulate proliferation, whereas exposures of long duration are cytotoxic. The proliferative response has been shown to be mediated through the medium. Results to date indicate that a growth-stimulatory substance(s) is produced in the amino acid and vitamin mix by exposure of the mix to visible light.

C. Methylation of Eukaryotic DNA (Dr. Gantt and coworkers). The locus of 5-methylcytosine, a ubiguitous minor base in DNA, is randomly distributed along the DNA chain with respect to the nucleosome. The hypothesis that nucleosome binding in eukaryotic DNA is determined by the locus of 5-methylcytosine was tested by isolating nuclei of chicken red blood cells, treatment of the nuclei with S-1 nuclease, and isolation of the DNA bound to the nucleosome monomer and the solubilized DNA. Analysis of these DNA fractions showed 5-methylcytosine to be distributed equally between them. The importance of nucleic acid methylation has recently been emphasized by the discovery of post-transcriptional mRNA modifications required for translation.

D. Methylases in the Intracisternal A Particle (Dr. Gantt and coworkers). The intracisternal A virus-like particle contains several RNA-methylating enzymes, as shown by rigorous particle purification and sedimentation in sucrose gradients after treatment of the purified particles with 1% triton X-100. The RNA bases modified include adenine, guanine, and cytosine. The presence of at least three RNA methylating enzymes in the core of the A particle suggests additional modes of post-transcriptional regulation of processes involving nucleic acids.

E. Role of Oxygen for Survival, Growth, and Metabolism of Sparse Cell Populations (Dr. Taylor and coworkers). The PO_2 of the culture medium was found to regulate proliferation rate within the limits tested (30 to 150 mm Hg) and the effect was density-dependent. Whereas lowering the PO_2 of the culture medium enhanced colony development and cell numbers from low inoculum sizes ($< 10^4$ cells/T-15), little enhancement was observed in mass cultures from high inoculum sizes. Glucose utilization and lactate production varied inversely with the initial inoculum size and oxygen concentration.

F. Analyses of a Peptide-Like Growth Factor for Mammalian Cells in Serum-Free Medium (Dr. Taylor and coworkers). Preliminary results suggest that fractionation of an antoclavable, low molecular weight fraction from peptone on a P-2 gel filtration system is superior to G-25 gel filtration and to CM-52 ion exchange columns. This factor promotes continuous proliferation of mouse, rabbit, hamster, monkey and human cell lines.

G. Procurement and Cultivation of Human Epithelial Cells (Dr. Taylor and F. Price with coworkers). A reproducible source of "normal" human tissue for epithelial cell culture has only recently become available. A culture medium designed specifically for human epithelial cell growth is being

formulated and routine production of such cultures has now been successful. This is the first phase of a program to attempt to transform human epithelial cells in culture and to study the changes at the nutritional, biochemical and morphologic levels.

H. Morphology of Transformation in Human and Rodent Cells (S. Handleman and Dr. Sanford). The objective of this project is to identify and quantify cytologic characteristics of cells in culture that are associated with their neoplastic transformation by chemical carcinogens as well as to follow the progressive subtle changes associated with their spontaneous transformation in culture. The cytology of rodent fibroblasts undergoing spontaneous transformation was analyzed and five of 19 properties were found to correlate with neoplastic transformation in all 13 cell lines, namely, increased cytoplasmic basophilia, reduced cytoplasmic spreading on substrate, cording, high nuclear:cytoplasmic ratio, and clumping. Studies were extended to epithelial cell types where successful cytologic diagnosis of neoplastic transformation and criteria for diagnosis were established.

(Drs. Fox and Sanford in collaboration with Dr. Caspersson and coworkers, Stockholm, Sweden). Populations from seven pairs of neoplastic and non-neoplastic rodent cell lines of common origin were compared for evidence of a change in quantifiable morphologic parameters accompanying neoplastic transformation. Neoplastic transformation in these and human fibroblasts (Drs. Fox, Kakunaka, Caspersson and associates) was consistently accompanied by three morphological features: decrease in projected area of the cytoplasm, decrease in projected area of the nucleus, and decrease in dry mass of the cytoplasm. In addition, protein concentration was increased. These results provide quantitative evidence that neoplastic transformation of both rodent and human fibroblasts in culture is accompanied by morphologic change.

I. The Topography of Cells in Culture (Dr. Sanford and coworkers in collaboration with Dr. B. Wetzel, Dermatology Branch, NCI). By combined autoradiography, scanning electron microscopy, and time-lapse photomicrography on CHO cells, it was found that the cell cycle exerts little influence on the topography of interphase cells in asynchronous culture.

J. Locomotor Behavior of Nonneoplastic and Neoplastic Fibroblasts in Culture (Dr. Sanford and coworkers in collaboration with Dr. R. Tarone). Cells in all cultures of nonneoplastic lines exhibited a "persistent" walk when measured over short intervals, whereas cells from the corresponding neoplastic derivatives had a random pattern of locomotion. These observations indicate a certain loss of polarity in the neoplastic cell possibly associated with the reduced lamellar cytoplasm, fewer attachment sites, and the higher migration rate.

V. CELLULAR REGULATION SECTION (Dr. R.L. Goldberger, Head)

In recent years an important approach has been developed for studying the molecular mechanisms by which hormones regulate gene expression in eukaryotic organisms. The goal of this approach is to study the regulatory effects of hormones in vitro, and involves the following steps: isolation of a specific messenger RNA (mRNA) from the target tissue after adminis-

tration of hormone; identification of this mRNA by its ability to program the synthesis of a specific protein in vitro; purification of the mRNA to homogeneity; use of the pure mRNA as a template for synthesis of complementary DNA; and, finally, use of the complementary DNA as a probe for hybridization studies of in vitro transcripts from purified components with RNA polymerase and chromatin. Studies successfully utilizing this approach have been restricted to specialized tissues in which the potential for gene expression is severely limited. We have recently begun a study along the general lines of this approach but have chosen to study specific regulation of gene expression in liver, a tissue with diverse genetic potential. Our studies involve an investigation of the induction of vitellogenin synthesis by estrogen in rooster liver.

Vitellogenin is the precursor of the chicken egg yolk phosphoproteins, phosvitins and lipovitellin. This protein is normally synthesized in the liver of the laying hen and is transported through the blood to the ovary, where it is deposited in the developing oocyte. Vitellogenin is not normally synthesized by immature chickens or by roosters, but its synthesis can be induced in these animals by estrogen. Finding no structure into which it can be deposited, vitellogenin accumulates in the plasma of the estrogen-stimulated rooster. While the vitellogenin gene is not transcribed in normal rooster liver, transcription of this gene following stimulation of the rooster with estrogen results in the synthesis and accumulation of so much vitellogenin mRNA that its presence is detectable in crude polysomal RNA.

One of the difficulties encountered in studying the vitellogenin system at the molecular level is that the protein is extremely large (monomer molecular weight 240,000) and hence vitellogenin mRNA is extremely large (about 7000 nucleotides long). Purification of such an enormous mRNA is made difficult by the fact that chicken liver is unusually rich in ribonucleases, and has required development of new techniques. Translation of vitellogenin mRNA into full-sized vitellogenin in a cell-free system derived from wheat germ has also required ingenuity, as such a large polypeptide has never before been successfully translated.

A. Purification and Translation of Vitellogenin mRNA and Synthesis of cDNA (Dr. R. Deeley and colleagues). Vitellogenin mRNA has been purified to homogeneity as determined by electrophoresis in agarose gels under denaturing conditions. Its size, estimated both from its electrophoretic mobility in agarose gels and contour length measurements in the electron microscope, has been determined to be 2.35×10^6 daltons. The homogeneity of the RNA preparations has also been assessed by studying the hybridization of vitellogenin cDNA to its RNA template. The results obtained were consistent with those expected for a homogeneous preparation containing a single species the size of vitellogenin mRNA. It has been possible to translate purified vitellogenin mRNA into a polypeptide the size of complete vitellogenin monomer (MW 240,000). In addition, the in vitro product has been shown to react with purified monospecific anti-vitellogenin antibody and to yield an extremely unusual peptide characteristic of vitellogenin on cleavage with cyanogen bromide. It has been demonstrated that this peptide (MW 90,000) contains the two phosphoserine rich-polypeptides found in egg yolk, the phosvitins, which are derived in vivo by

specific proteolysis of vitellogenin. Experiments designed to measure the kinetics of synthesis of vitellogenin in vitro have been used to localize the phosvitins in the COOH-terminal portion of the vitellogenin polypeptide. The size estimates of vitellogenin mRNA indicate that it is approximately 10% larger than the minimum size required to specify the vitellogenin polypeptide.

Radioactive complementary DNA synthesized from purified vitellogenin mRNA has been used as a sensitive and direct means of measuring the concentration of vitellogenin mRNA in rooster liver, both prior to treatment with estrogen and at various times after treatment. It was found that the livers of normal roosters contained 0-5 molecules of vitellogenin mRNA per cell. Following a single injection of estradiol, the concentration of vitellogenin mRNA increased linearly for 24 hours and reached a maximum concentration (at 3 days) of approximately 10,000 molecules per cell. Thereafter, the concentration declined, decreasing to approximately 400 molecules per cell at 10 days and to less than 100 molecules per cell at 14 days. Thus, the induction of vitellogenin synthesis in the rooster appears to involve primary activation of a previously dormant gene in a tissue that is already differentiated and metabolically active. This activation process is not accompanied by the cellular proliferation found in other systems regulated by steroid hormones and which exhibit a response of this magnitude, such as the synthesis of ovalbumin in the chicken oviduct.

B. Development of in Vitro Systems to Study Transcription of the Vitellogenin Gene (Dr. K. Mullinix and colleagues). Nuclei isolated by our procedures from rooster liver are pure as measured by phase contrast microscopy and are very active in transcription. The conditions employed for RNA synthesis involve the use of relatively high levels of nucleotide triphosphates, a triphosphate generating system (phosphocreatine and phosphocreatine kinase) and rat liver cytosol ribonuclease inhibitor. The inhibitor is necessary to retard the breakdown of RNA due to the very high levels of ribonuclease present in preparations of rooster liver nuclei. The synthesis of RNA is linear for approximately 30 minutes. Both DNA dependent RNA polymerases I and II are active in our preparations, 62% of the synthesis being due to RNA polymerase II as measured by the sensitivity of this synthesis to α-amanitin. The nuclei incorporate approximately 4 picomoles of UMP into RNA per μg of nuclear DNA in 10 minutes of incubation at 25°C, a level of synthesis consistent with some reported in the literature for nuclei from other sources. We have found that nuclei from estrogen-treated rooster liver are approximately twice as active in transcription as those from livers of unstimulated roosters. We have had considerable difficulty in isolating high molecular weight RNA from our preparations since the levels of ribonuclease in these nuclei are so high. We are still in the process of trying to overcome this problem. Chromatin isolated from rooster liver nuclei by our procedures is also very active in transcription utilizing the endogenous chromatin-associated RNA polymerases. The chromatin directs RNA synthesis to the extent of incorporation of 5 pmoles of UMP into RNA after 10 minutes of incubation at 25°C. This synthesis is 50% inhibited by α-amanitin.

We have begun to assess methods to monitor RNA that is synthesized in vitro. The methods that we have considered involve isolation of RNA and hybridization of the isolated RNA to complementary DNA (cDNA) made to purified

vitellogenin mRNA. We have labeled RNA synthesized in vitro with ^{3}H-UTP and have purified RNA from nuclei. By this approach, endogenous RNA and newly synthesized RNA are present in the final product. In addition, we have labeled newly synthesized RNA with both ^{3}H-UTP and Hg-CTP. Following the synthesis, RNA is deproteinized and the RNA that was synthesized in vitro is isolated by chromatography on sulfhydryl-sepharose columns under conditions in which mercury-labeled RNA binds to the support whereas endogenous RNA does not bind. The mercury-labeled RNA is then eluted from the support, thus yielding a fraction of RNA containing only newly synthesized RNA. The latter method has the advantage of theoretically being able to separate RNA synthesized de novo from endogenous RNA, but we have found technical problems in its implementation.

As an approach toward reconstructing a transcription system utilizing the vitellogenin gene DNA, RNA polymerase, and other necessary proteins, we have solubilized RNA polymerase from lysed nuclei by digesting DNA with DNAse bound to sepharose, liberating the RNA polymerase from its template. Preliminary data suggest that this is an efficient method of solubilizing both RNA polymerases I and II. These enzymes are active in transcription and utilize native DNA as the preferred template.

C. Organization of Estrogen Responsive Genes in the Avian Genome (Dr. F. Eden and colleagues). The aim of this project is to determine the actual number of different structural genes in avian liver whose transcription is altered by administration of estrogen and to provide a detailed description of the organization of these estrogen-responsive genes in the avian genome.

To determine the prominent features of sequence organization in chicken DNA, the reassociation kinetics of short fragments of chicken DNA were measured by hydroxyapatite chromatography. Chicken DNA contains repetitive and single copy DNA sequences, as do most eukaryotice genomes. To study the arrangement of these sequences, short, unlabeled fragments of chicken DNA were reassociated with trace amounts of radioactive fragments ranging in size from 200 nucleotides (nt) to 18,000 nt in length. The hydroxyapatite binding of radioactive fragments of various lengths was determined after reassociation of repetitive sequence elements. An increase in hydroxyapatite binding via repetitive sequences as a function of tracer fragment length provides direct evidence for interspersion of repetitive and single copy sequence elements and indicates what fraction of the genome is interspersed as well as the average spacing between repeated sequences. Using this information, the chicken genome was preparatively fractionated into single copy and repetitive sequences. The single copy DNA fraction was labeled in vitro to high specific activity and used in the studies described below.

To quantitate the number of structural genes expressed in livers of roosters that have not been treated with estrogen, total RNA was extracted from the livers of 6-8 week old roosters. A second group of roosters received a series of injections of estradiol and when vitellogenin synthesis reached maximal levels, the livers were excised and RNA isolated as above. Each total RNA preparation was hybridized with single copy DNA, using an RNA excess of 100,000-fold. From the saturation plateau of the hybridization reactions,

we can calculate directly the number of structural genes expressed in stimulated and unstimulated liver.

From the reassociation kinetics of short fragments of chicken DNA measured by hydroxyapatite chromatography we have determined that 10-12% of chicken DNA sequences are repeated about 200-fold. The remaining 88-90% of chicken DNA is composed of single copy sequences. From the reassociation kinetics of an isolated single copy tracer fraction with excess total DNA we calculate that the kinetic complexity of chicken single copy DNA is 2.1 pg, or about 2×10^9 ntp. Thus, the chicken genome contains sufficient single copy sequence to code for about 10^6 structural genes, each 2000 ntp in length.

From the hydroxyapatite binding of DNA fragments of various lengths via repetitive duplexes we have determined that about 40% of the chicken genome bears an alternating arrangement of single copy and repetitive sequences with an average spacing of about 4000 ntp. An additional 10-15% of the genome appears to contain a wider spacing of repetitive sequence elements. The remaining 45-50% of the genome consists of single copy sequences uninterrupted by repetitive sequence elements for a distance of at least 18,000 ntp. Preliminary observations, using partially reassociated 7000 ntp fragments of chicken DNA examined with the electron microscope, confirm these general features of sequence organization.

We are presently isolating those single copy sequences that are most closely interspersed with repetitive sequence elements. These sequences should constitute a small fraction of the total sequence complexity and will be used to determine whether a functionally related subset of single copy sequences -- the estrogen-responsive genes in rooster liver -- enjoy a preferential location adjacent to repetitive sequences. This information will indicate whether proximity to repeated sequences plays a role in the coordinated expression of estrogen-responsive genes.

VI. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. M. F. Singer, Head)

A. Polynucleotide Phosphorylase and Cyclic AMP Phosphodiesterase. Claude Klee and her colleagues, taking Efraim Racker's dictum, "Don't waste clean thoughts on dirty enzymes", to heart, have purified, to homogeneity, the various proteins in which they are interested, and their clean and clear thoughts have operated upon the pure proteins to interesting advantage. The decade of mystery surrounding the multiple forms of M. luteus polynucleotide phosphorylase has come to an end. The predominant species is an oligomer of three identical subunits with a combined molecular weight of 250,000. The oligomer can exist in multiple conformational states, separable on electrophoresis, and inducible by the binding of oligo and polynucleotides. The enzyme can also exist as a complex of 400,000 daltons containing another polypeptide (Elizabeth Barbehenn). Protein-protein interactions are also central to Dr. Klee's interest in the regulation of cAMP phosphodiesterase. The enzyme itself has been purified, as has the protein that activates the enzyme in the presence of calcium ion and a third protein that acts as a specific inhibitor of the activation. Study of the nitration of the activator protein at tyrosine residues has shown that the conformation is significantly altered by the binding of calcium ion (Paul Richman). In

view of the similarity of the activator protein with the calcium-binding component of the troponin complex, troponin C, the relationship of the cyclic phosphodiesterase system to the contractile proteins of the cell is also being investigated.

B. Binding of H1 Histone to DNA (Dr. D. Singer). The basis for the preferential complexing of superhelical DNA as compared to relaxed DNA, by H1 histone, is now understood. While the relaxed DNA undergoes an exclusively cooperative interaction with H1 (40-45 molecules of H1 per molecule of relaxed SV40 DNA), superhelical DNA can noncooperatively form complexes containing only 10-12 H1 molecules per DNA, as well as complexes containing 45-80 molecules of H1 per DNA, cooperatively. Phosphorylating H1 at various specific sites does not significantly alter the modes of interaction.

C. Effects of H3 and H4 Histones on the Physical State of DNA (Dr. M. Bina-Stein). Utilizing enzymic, hydrodynamic, and electron microscopic techniques, H3 and H4 histones alone were found to compact duplex DNA and introduce torsional constraints equivalent to that obtained with the full complement of histones H3, H4, H2a and H2b, in intact nucleosomes.

D. Effect of SV40 Infection on Histone Synthesis (Dr. A. Kay). The synthesis of histones of all five classes is stimulated to about the same extent after infection of monkey kidney cells with SV40. However, the rate and timing of histone synthesis is not strictly coupled to SV40 DNA synthesis, as might have been expected from the usual observation of strict coupling of histone and cellular DNA synthesis.

E. Genomic Differences between Two Wild Type SV40 Strains (Dr. A. Kay). The genomes of two known wild type strains of SV40 were shown to differ significantly in several areas of the DNA and most markedly in the region coding for the major capsid protein. The observed difference in DNA was confirmed by differences in the tryptic peptides produced from the capsid proteins of the two strains.

F. Sequence Analysis of Defective SV40 DNA and Highly Repetitive Monkey DNA (Dr. H. Rosenberg, Dr. S. Segal, Dr. E. Kuff [Biochemistry Section] and Dr. M. Rosenberg [Laboratory of Molecular Biology)]. Several years of study of a defective variant of SV40 that contains monkey sequences in place of some viral sequences has finally yielded the opportunities we had hoped were inherent in this defective variant. The nucleotide sequence of two DNA segments has been determined. One sequence of 185 base pairs, isolated from the defective virus, shares 156 residues in common with a 172 base pair long fragment, isolated directly from monkey DNA. The latter fragment is repeated almost two million times in the monkey genome, very often in tandem array. However, within the 172 base pair monkey sequence there are no internally repetitious sequences. At either end of the 156 residues within the defective that have been identified as originating from the monkey DNA, the sequence joins other sequences of uncertain origin.

VII. PROTEIN CHEMISTRY SECTION (Dr. E. Peterson, Head)

A. Chromosome Transfer (Dr. O. W. McBride and colleagues). In an attempt to estimate the size of the DNA segment transferred during biological transformations of cells with isolated chromosomes, hybridization techniques have been utilized to isolate radiochemically-labeled human chromosomal donor DNA (transgenome) from a clonal line of mouse cells containing the transferred hprt gene. This labeled DNA probe was evaluated by its reassociation in the presence of driver DNA from various sources, including mouse (recipient), human (donor), and the transformed clone (transgenote). The resulting data suggest that the transgenome represents 40-80% of an intact human X-chromosome. This surprisingly large value appears to be inconsistent with the failure to detect any of three different nonselected donor X-chromosomal protein markers in the transgenote. It could be explained, however, if multiple donor chromosome fragments or multiple copies of the same fragment are present in the transgenote, or if there is no regulatory control for transcription of the transgenome.

In preparation for additional investigation of the size and mode of integration of the transgenome into transgenotes, the human gene for thymidine kinase has been transferred to mouse TK^- cells and the human hprt gene to Chinese hamster cells and to chloramphenicol-resistant mouse cells. Also, human-mouse and Chinese hamster-mouse hybrids have been obtained by cell fusion, and segregants are being isolated to contain, in a mouse background, only the human X-chromosome or human chromosome 17 (which contains the TK locus) or a single intact hamster chromosome bearing the TK and galactokinase genes.

B. Granulocyte Development (Dr. W. Evans and colleagues). A granulocytic leukemia has been developed in guinea pigs, the first to be reported in this species. This leukemia, which appeared initially in one of forty strain 13/N females given N-nitroso-N-butylurea continuously in their water for 21 weeks, has been transplanted in this strain for 13 transplant generations by intraperitoneal inoculation of leukemic blood or marrow cells. Macroscopically and microscopically it resembles the chronic myelogenous form in human beings. However, unlike human leukemic cells, these are alkaline phosphatase positive. Electron microscopy revealed the presence of numerous intracisternal A-type particles that are not found in normal leukocytes. In collaboration with Dr. Mage, antisera specific for several granule proteins have been prepared for use in assays for regulators of granulocyte production in normal, inflammatory, and leukemic guinea pig bone marrow. One of these antisera was shown by the immunofluorescent procedure to have a high degree of specificity for neutrophils in the presence of other bone marrow cells.

C. Cellular Immune Reactions: Cell Separations (Dr. M. Mage and associates). Mouse spleen cells without surface immunoglobulin (Ig^- cells) have been further characterized. Such cells retain graft *vs* host activity and give the mixed lymphocyte reaction. They respond to the T-cell mitogens, concanavalin A and PHA, and they generate cytotoxic effector cells *in vitro*. An unexpected finding is that these Ig^- populations can generate antibody plaque-forming cells *in vitro* -- that is, they probably contain the precursors of B-cells that have not yet produced detectable surface Ig.

A new general method of cell separation based on the binding of antibody-coated cells to anti-immunoglobulin-coated surfaces has effected the separation of Ig^- from Ig^+ spleen cells and the separation of mature neutrophils and their precursors from other cell types in guinea pig bone marrow.

A guinea pig antibody to mouse cytotoxic effector cells and their precursors has been prepared. It appears to have minimal effect on several other cellular immune reactions and is being used to help delineate the subtypes of T-cells that participate in graft vs host and other immune reactions.

D. Displacement Chromatography of Proteins (Drs. E. Peterson and R. Torres). A study of displacement chromatography of proteins, using relatively simple mixtures of commercially purified proteins has continued. The shapes of the major protein bands in the emerging displacement trains were in agreement with displacement theory, and the system was capable of resolving very similar proteins at relatively high loads. A procedure for concentrating dilute proteins by adsorption to an anion exchanger, followed by displacement with CM-dextran has been developed. Unlike the concentrated product of similar procedures based on sharp elution of the adsorbed protein with salt solutions, the concentrate obtained by displacement can be subjected directly to electrophoretic analysis.

The capacity of DEAE-cellulose for binding CM-dextrans in the immobilized range was found to be, on a mass basis, inversely proportional to their content of carboxymethyl groups and, consequently, inversely proportional to their affinity for the column. This finding, which is in contrast with the adsorption behavior of proteins, permits the selection of appropriate relative concentrations for successive sections of the continuum of displacers used in separating complex mixtures of proteins.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 5210-09 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cellular Controls over Growth and Inducible Processes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The nature of cellular controls over responses to steroid hormones has been studied through somatic cell hybrids. Processes studied in these hybrids include induction of tyrosine aminotransferase, prolactin, growth hormone and glutamine synthetase and inhibition of nucleic acid uptake, cell growth and cloning efficiency. Steroid-resistant cell variants have been developed. Characterization and purification of glucocorticoid receptors are underway. Receptors for glucocorticoids, androgens, estrogens and progestins, have been studied for their correlation with treatment and disease in human leukemias and breast cancer. Growth control in cell hybrids has been studied and hybrid membrane proteins analyzed. A line of human leukemic cells has been characterized with respect to steroid responsiveness and will be utilized to study the cell genetics of steroid resistance, the mechanism of steroidal lymphocytolysis, and the effects of combined drug therapy in vitro.

PHS-6040
(Rev. 10-76)

Cooperating Units (Cont'd) A. Kandutsch and H. L. Chen, The Jackson Laboratory; T. V. Gopalakrishnan and F. Anderson, H/IR/MH; R. Ader and J. Cohen, A/LCP; K. Kim, I/LMI; M. Rosenberg, C/LMB; E. Kuff, C/LB; D. K. Granner, U. Iowa Medical School; T. D. Geleherter, U. Michigan Medical School; D. Salomen, D/LDBA; J. O. Bishop, U. Edinburgh.

Project Description:

Objectives: Understanding gene expression in eukaryotes remains our overall objective, and the major systems in which we are studying this problem continue to be those under the control of steroid hormones. In addition, we have continued to try to apply these basic results to pertinent clinical situations. As before, some additional studies have been carried out as the result of opportunities of the moment, not directly related to the action of steroids, but nevertheless pertinent to eukaryote gene control systems.

First, this report will review the objectives outlined in last year's annual report. Some of these have been terminated. Others remain as current goals. Our first objective still is to define at the cellular level, controls over responses to steroids. Related to this, our second objective last year was to develop cell variants with respect to steroid responsiveness.

The third objective last year was to seek evidence for cytoplasmic factors affecting gene expression. This objective has been met so that work in this area has been terminated (see Findings and Publications).

The fourth objective last year was to seek cellular receptors for oxygenated steroids, analogous to those for steroids. This work was also successful (see Findings) and has been terminated.

Last year's fifth objective was to see whether steroid receptors were involved in the stimulation by glucocorticoids of C-type virus induction by 5-iodo-2-deoxyuridene. Because of altered personnel, this project has been terminated without conclusive results.

The sixth objective was to seek purification and characterization of glucocorticoid receptors and to compare their properties with other classes of receptors. These studies are actively being pursued.

Seventh, we wished to study the steroid receptors in various blood dyscrasias and in breast cancer, to examine their role in the biology of the diseases and their predictive value in planning steroid therapy. This work continues. Two other clinically related projects have been terminated. Namely, the work on the estrogenization seen after treatment with spironolactone and cardiac glycosides has been completed and published. The studies using nuclear magnetic resonance to distinguish normal and malignant cells have also been terminated.

Eighth, we initiated studies on mutagenesis and transformation in xeroderma pigmentosum (XP) cells in vitro.

In summary, objectives which are being continued are:

1) To define steroid controls at the cell level;

2) Development of cell variants for steroid action;

3) Characterization and purification of glucocorticoid receptors;

4) To correlate steroid receptors in blood dyscrasias and breast cancer with the biology and treatment of the diseases;

5) To see whether XP cells can be transformed in vitro by UV light and to measure UV mutagenesis in vitro.

To these the following new objectives have been added:

6) To determine the levels of translatable tyrosine aminotransferase (TAT) mRNA under various conditions of hormonal treatment in inducible cells;

7) To obtain a similar in vitro translation assay for prolactin mRNA, and to isolate prolactin mRNA, prepare its cDNA, and use these reagents to quantify the mRNA in GH_3 and other cells after hormonal stimulation.

8) To characterize the mRNA populations during myogenesis in embryonic chick muscle. Work now is terminated.

9) Collaborations with other groups, described in more detail in their reports, include translation of A particle RNA in vitro (with E. Kuff), translation of AMV RNA in vitro (with D. Marciani and T. Papas), and translation of "capped" vs "uncapped" (i.e. 5'-methyl-G) prokaryotic mRNA's in vitro. We also have carried out work (with D. Salomen) on the ontogeny of glucocorticoid receptors in inbred mice exhibiting high or low teratogenesis after steroid treatment in utero.

Methods Employed: In addition to those previously given, methods employed included translation of guanidine HCl-extracted cellular RNA's and other RNA's in the wheat germ and reticulocyte systems, acrylamide gel electrophoresis, chromatography on ion exchange resins, and on various molecular seiving columns, and nuclear binding assays of steroid receptors.

Mutagenesis has been carried out with a variety of chemical mutagens and with X-rays as well as UV light. Nude (athymic) mice have been injected with various mutagenized cells and tumor cells.

Major Findings:

Objectives 1 and 2: Analysis of our data on HTC x L cell hybrids is very suggestive that the "inhibitory" responses of the L cell, like the induction of tyrosine aminotransferase (TAT) in the HTC cell, is recessive in early hybrids. However, the data are not yet firm enough for publication, and additional experiments, perhaps using some of our new cell lines (see below), will be necessary to confirm or deny these results.

Hybrids between the prolactin-producing GH_3 cells and L cells show loss of prolactin synthesis early after hybridization. This peptide is induced in the GH_3 cell by estrogen or TRH, but not in the hybrid. Thus it seems that: 1) The estrogen-induced function seems recessive in the hybrids, adding to the view that there may be universal specific inhibitory controls in cells; 2) responsiveness to the membrane-active inducing tripeptide TRH also is lost. This we believe to be the first report suggesting the existence of such cellular controls. In addition, one hybrid clone, after loss of numerous chromosomes, was found to be an unusually high producer of prolactin. We hope to utilize these cells in our work on prolactin mRNA (objective 7).

With regard to developing new cell variants, we have published a description of several clones of HTC cells not inducible for tyrosine aminotransferase but possessing normal glucocorticoid receptors. These clones are now being analyzed for several other steroid-inducible functions and for their TAT translatable mRNA content.

The line of CEM cells has been characterized with respect to its steroid responses. Several clones of CEM cells have been isolated which are either killed by steroids or which are resistant to killing. Dose-response data, steroid specificity, and receptor occupancy have been defined. By flow microfluorometry it has been shown that the cells are trapped in G1 by steroids. These data are being prepared for publication. With these CEM cells, we hope to study the genetics of steroid resistance, the biochemistry of steroid killing, and the effects of multidrug therapy in vitro.

Regarding objective 3, the characterization and purification of glucocorticoid receptors, we have preliminary evidence that we have separated, in crude form, the "inactive" from the "activated" form of the steroid-receptor complex. We have confirmed Milgrom's recent report of a small heat-stable inhibitor of activation and are attempting its purification. The two forms of receptor were reported on at the Federation Meetings and a paper has been submitted about this work.

Objective 4: Steroid receptors in patients with blood dyscrasias and breast cancer. Five patients with Sezary's syndrome have now been analyzed. As with acute lymphoblastic leukemia, there appears to be a correlation between the presence of receptors in the Sezary cells and response to steroid therapy. Sezary cells have been shown by Broder et al. to be

helper T cells. As a model system, therefore, we have examined established lines of mouse lymphoid cells with helper or suppressor T cell activity for steroid receptors. Initial results show a marked difference in receptor content in the lines. We plan to pursue the functional relevance of this finding.

More than 650 breast cancer patients have been analyzed for several steroid receptors. We have found several patterns of receptor content and are collating the clinical data preparatory to preparing a manuscript. In 11 male patients with breast cancer, estrogen receptors have been found. This observation is being prepared for publication.

Objective 5 has been pursued, with promising initial results. First, preliminary experiments on the xeroderma pigmentosum cells suggest that a ouabain selection system can be used to measure ultraviolet light-induced mutagenesis in normal and XP cells. Second, work has begun to determine if ultraviolet light-induced *in vitro* transformation of cultured XP cells can be observed by screening irradiated cells for their ability to produce tumors in nude mice. Finally, the relationship between survival of ultraviolet-irradiated cells *in vitro* and the clinical characteristics of the patients from which the cell strains are derived is being studied by cloning those strains which have unusual survival characteristics as well as unusual clinical symptoms.

Objective 6, to measure translatable TAT mRNA during hormone induction, has no major findings as yet, but the assay system to do so seems nearly ready to use.

Objective 7, to study prolactin mRNA, has just begun. As mentioned above, we have found that one clone of GH_3 x L cell hybrids produce unusually high levels of prolactin. We seek to use these cells as a source of mRNA.

Objective 8, characterization of the mRNA population during myogenesis in cultures of embryonic chick breast muscle. The sequence complexity, abundance class distribution and coding capacity of the mRNA has been determined as myogenesis proceeds. The work was done in collaboration with Dr. J. O. Bishop, University of Edinburgh, Edinburgh, Scotland, and has been submitted for publication.

Objective 9, the role of the 5' methyl-G or "cap" structure in the efficient translation of defined prokaryotic mRNAs in eukaryotic cell-free systems. In collaboration with Dr. M. Rosenberg, C/LMB, we have been able to translate several bacteriophage and E. Coli mRNAs in various eukaryotic cell-free systems and have defined the parameters necessary to achieve efficient translation. We are now in the process of examining initiation events in protein synthesis in relation to the cap structure, i.e., ribosome binding, formation of the initiation complex, and sequence of the ribosome binding site on defined prokaryotic mRNAs. The first part of this work is being prepared for publication.

Identification of A particles as endogenous viral particles in the mouse. This work is being done in collaboration with Dr. E. Kuff. We have shown that RNA isolated from A particles codes for the major A particle structural protein, suggesting that these particles are true viruses. The work is in preparation for publication.

Arrangement and processing of the structural genes on AMV. This is being done in collaboration with Drs. D. Marciani and T. Papas, C/LTVG. We have been able to synthesize specific AMV viral polypeptides in vitro and are looking at the structural gene sets contained in each polypeptide by tryptic peptide analysis. This pattern will be compared with the putative processing scheme characterized for viral processing in vivo. After completion of the tryptic maps, this work will be ready for publication.

The work on the ontogeny of glucocorticoid receptors (Y. Zubairi and D. Salomen) also has been completed and submitted for publication.

Findings from Objectives Now Terminated:

Cytoplasmic factors in L cells and neuroblastoma cells have been shown to permanently suppress the synthesis of hemoglobin and its induction in erythroleukemic cell lines. This was demonstrated by forming cybrids between the two cell types. (Published)

Preliminary data suggesting the existence of receptors for oxygenated steroids has been accepted for publication. (Authors Drs. Kandutsch and Chen. Acknowledgment to Dr. Thompson.)

Evidence linking the occupancy of androgen receptors by the non-androgen spironolactone with the estrogenizing effects of this anti-mineralocorticoid has been published.

Proposed Course of Research

We plan to pursue three main areas of research. First will be the area of somatic cell genetics. In this area we will try to see whether the development of steroid resistance by CEM cells follows genetic rules. Selective markers will be placed in CEM cells, and dominance/recessive and complementation tests carried out by somatic cell hybridization. The HTC cell variants will be characterized and similarly studied. If we can obtain the skilled individual necessary to do the karyology, we will try to map three human genes: those for the glucocorticoid receptor, for the repression of TAT induction, and for the regulatory subunit of protein kinase.

Second, we hope to purify the inhibitor of activation of the glucocorticoid-receptor complex.

Third, we hope to define the kinetics of induction and deinduction of functional TAT mRNA. Vis a vis prolactin, we hope to be able to prepare

a specific cDNA and use it to define the changes in annealable prolactin mRNA after treatment of sensitive cells with glucocorticoids, estrogens, and TRH.

In addition, we will continue our clinical correlation studies with steroid receptors, and on the work with suppressor and helper T cell lines, to see whether steroids have different effects on them.

We plan to terminate the work on AMV sequencing, A particle RNA, "capping" and prokaryote RNA's as these pieces of work are completed or are nearing completion.

Publications

Yang, S. S., Chan, C., and Thompson, E. B.: Rescue of a rat tropic rat hepatoma virus pseudotype kirsten sarcoma virus by co-cultivation of hepatoma tissue culture cells with K-NRK cells. J. Gen. Virol. 31: 239-243, 1976.

Pita, J. C., Jr., Lippman, M. E., Thompson, E. B., and Loriaux, D. L.: Interaction of spironolactone and digitalis with the 5α-dihydrotestosterone (DHT) receptor of rat ventral prostate. Endocrinology 97: 1521-1527, 1975.

Thompson, E. B., Perlin, E., Tormey, D.: Steroid-binding proteins in carcinoma of the human male breast. Am. J. Clin. Pathol. 65: 360-363, 1976.

Marciani, D. J., Lyons, L. B., and Thompson, E. B.: Characteristics of cell membranes from somatic cell hybrids between rat hepatoma and mouse L-cells. Cancer Res. 36: 2937-2944, 1976.

Lyons, Linda B., and Thompson, E. B.: Delayed malignancy and altered growth properties of somatic cell hybrids between rat hepatoma and mouse L-cells. J. Cell. Physiol. 90: 179-192, 1977.

Thompson, E. B., Aviv, D., and Lippman, M. E.: Variants of HTC cells with low tyrosine aminotransferase inducibility and apparently normal glucocorticoid receptors. Endocrinology 100: 406-419, 1977.

Gopalakrishnan, T. V., and Thompson, E. B.: Effect of Concanavalin A on tyrosine aminotransferase in rat hepatoma tissue culture cells. J. Biol. Chem. 252: 2717-2715, 1977.

Gopalakrishnan, T. V., Thompson, E. B., and Anderson, W. F.: Extinction of hemoglobin inducibility in Friend erythroleukemia cells by fusion with cytoplasm of enucleated mouse neuroblastoma or fibroblast cells. Proc. Natl. Acad. Sci. USA 74: 1642-1646, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 366-07 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biosynthesis and assembly of intracellular components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	K. K. Lueders	Chemist	LB NCI
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COOPERATING UNITS (if any)

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3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying murine intracisternal Type-A particles as a model for the expression of integrated viral genes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the mechanisms for coordinately regulating the synthesis individual macromolecules and their assembly into complex intracellular components (organelles and viruses); to study the process of neoplastic transformation in terms of these coordinated functions.

Methods Employed: Tissue culture; isolation of subcellular organelles and viruses; labeling of nucleic acids and proteins in vivo and in cell-free systems; isolation and fractionation of nucleic acids using techniques of affinity chromatography, density gradient centrifugation and gel electrophoresis; estimation of genetic complexity and gene copy number by quantitative nucleic acid hybridization; chromosomal localization of specific DNA sequences by in situ hybridization; chromatographic, immunochemical and electrophoretic analysis of specific proteins.

Results: During the past year, we have continued our studies of murine intracisternal A-particles, with particular emphasis on characterizing the genetic information associated with these virus-like structures.

1. Polyadenylated RNA from myeloma A-particles was active as a template for protein synthesis in cell-free systems derived from both wheat germ and reticulocytes. RNA molecules 24S and larger coded in vitro for 73,000 dalton products which were identified as the main A-particle structural protein (P73) on the basis of their methionine-containing tryptic peptides. The RNA also directed the synthesis of 45,000 and 30,000 dalton peptides which had different methionine-peptide maps; these could represent previously unrecognized "non-virion" proteins which are present elsewhere in the particle-producing cells. The finding that A-particles contain RNA specifying their own structural protein is consistent with their being an intracellular form of virus. (Paterson, Segal, Lueders, Kuff)

2. We have identified a set of polyadenylated RNA sequences which are much more abundant in A-particle producing cells than in particle-negative cells and which are concentrated in A-particles isolated from three widely different tumor cell types (myeloma, neuroblastoma and rhabdomyosarcoma). This group of sequences has a genetic complexity of at least 5000 nucleotides. (Lueders, Segal and Kuff)

3. The A-particle associated sequences were found to be reiterated between 2000 and 4000 times in the genome of normal mouse cells (liver and sperm). The copy number was not significantly higher in A-particle producing cells. These sequences constituted about 25% of 30S polyadenylated RNA from neuroblastoma A-particles; the remainder consisted of RNA transcribed from sequences that were much less highly reiterated in the mouse DNA (fewer than 160 copies per cell). In situ hybridization of mouse chromosomes was performed using ^{3}H-cDNA representing the highly reiterated group of A-particle sequences: these sequences appeared to have a widespread non-centromeric distribution as well as being concentrated in definite regions of certain chromosomes. (Lueders and Kuff)

Proposed Course of Research: We intend to explore in more detail the relationship between the A-particle structural genes and the more highly reiterated class of A-particle associated sequences. Repetitive sequence elements are thought to have roles in regulating structural gene expression. Intracisternal A-particles, which are produced transiently in early mouse embryogenesis and reappear in many transformed cells, may be a useful model system in which to study this type of regulatory mechanism.

Publications:

Kuff, E.L., Lueders, K.K., Orenstein, J.M., and Wilson, S.H.: Differential response of type C and intracisternal type A particle markers in cells treated with iododeoxyuridine and dexamethasone. J. Virology 19: 709-716, 1976.

Lueders, K.K., Segal, S., and Kuff, E.L.: RNA sequences specifically associated with mouse intracisternal A-particles. Cell 11: 815-826, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 379-16 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Ultrastructural characterization of macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

CRP, a component of serum that increases dramatically in inflammation, has been shown to consist of five protomers arranged in cyclic symmetry at the vertices of a regular pentagon. Human and rabbit CRP measure 11 nM in width and the individual protomers are 3.8 nM in diameter. A molecular weight of 23,700 can be calculated. Pentameric molecules are so rare that a new class of molecules called PENTRAXINS has been proposed.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To relate the fine structure of macromolecules to their biochemical and physical properties, and to their function in biological systems.

Methods and Major Findings:

1. Ultrastructure of complement components, C5-9, the "membrane attack complex".

This project was terminated during the past year. It was concluded that methods for purifying the components of the complex were not good enough to provide samples that could be analyzed in the electron microscope.

2. Ultrastructure of c-reactive protein, CRP.

CRP, a component or serum that increases dramatically in inflammation, has been shown to consist of five protomers arranged in cyclic symmetry at the vertices of a regular pentagon. Although the molecules occasionally form stacks of two units, most molecules appear singly and it is concluded that they consist of 5, not 10, protomers. Human and rabbit CRP have the same dimensions within the limits of accuracy of measurement, 11 nM in width. The average diameter of the individual protomers is 3.8 nM. From these data a molecular weight of 23,700 can be calculated. It agrees well with a MW 22,900 found by sedimentation techniques.

Pentameric molecules are so rare that a new class called PENTRAXINS has been proposed. The class is composed of the three closely related pentameric molecules, CRP, amyloid P-copmponent and C1t.

This project was terminated during the past year.

3. Ultrastructure of stable antigen-antibody complexes

Plotz and Guyer are preparing stable antigen-antibody complexes which can be used as probes to visualize the site where immune complexes "home" in the body. The antigen, para nitrophenol, after reaction with specific antibody is crosslinked in the binding site forming a stable complex. The usual antigen-antibody complexes follow the law of mass action and are not useful as probes.

The preparation and purity of the complexes and their ultimate form is being studied by electron microscopy. As yet no uniform purity or structure has been achieved.

Significance to Cancer Research: A knowledge of the structure of macromolecules obtained by observations with the electron microscope provides confirmation of physico-chemical data and, in many cases, an unequivocal basis for knowledge of their function.

Proposed Course of Research: To elucidate further the structure of macromolecules and correlate their structure with their function.

Publications:

Osmand, A.P., Friedenson, B., Gewurz, H., Painter, R.H., Hofman, T, and Shelton, E.: Characterization of c-reactive protein and the complement component C1t as homologous proteins displaying cyclic pentameric symmetry (pentraxins). Proc. Nat. Acad. Sci. USA 74: 739-743, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 945-04 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study of the Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Peterkofsky	Research Chemist	LB NCI
OTHER:	R. Hata	Visiting Fellow	LB NCI
	D. K. Kalwinsky	Research Associate	LB NCI
	J. H. Eichhorn	Research Associate	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5-1/4	3-1/4	2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies are concerned with various aspects of collagen metabolism. Prolyl and lysyl hydroxylases, enzymes which catalyze the hydroxylation of prolyl and lysyl residues in collagen during synthesis of this protein on polysomes bound to the membrane of the endoplasmic reticulum, require Fe^{++}, α-ketoglutarate, oxygen and a reducing compound. Ascorbic acid is the most efficient reducing compound but others such as tetrahydropterins, tetrahydrofolate, dithiothreitol and cysteine are also active to varying extents. The localization of these enzymes in microsomes as well as the specificity of the reducing compound are being studied. Since hydroxylation of prolyl residues in collagen is required for collagen secretion while hydroxylysine is required for the formation of crosslinks to form mature collagen fibers, the enzymes and their cofactors may play a role in the regulation of collagen synthesis and the maintenance of connective tissue. Collagen synthesis is also being used as a marker to study the effects of neoplastic transformation of a differentiated cell function.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

1. Proteins of whole chick embryos, isolated bones of these embryos, or cell-free systems derived from these bones or cultured fibroblasts are labeled with ^{14}C-proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and non-collagenous proteins by a method involving specific digestion of collagen in a mixture of proteins using highly purified collagenase. This method was developed in our laboratory and is described in a previous report (1968-9). The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissue or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured fibroblasts are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

2. Cell-free incorporation of ^{14}C-proline is studied with a system consisting of membrane-bound or free polysomes derived from chick embryo bone tissue and soluble factors derived from chick embryo liver, a tissue which synthesizes very little collagen, so that variations in collagen synthesizing polysomes of a tissue may be studied independently of possible variations in the soluble fraction.

3. To determine the level of proline hydroxylation in collagen of cells or tissues labeled with radioactive proline, collagenase digests are hydrolyzed with 6N HCl and radioactive proline and hydroxyproline in the hydrolyzates are measured by a specific radioassay. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

4. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α,α'-dipyridyl, which inhibits hydroxylation.

5. Tetrahydropteridines in cell extracts were measured using purified phenylalanine hydroxylase which has a specific requirement for this cofactor.

6. Collagen types synthesized in cultured cells were determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using carboxymethyl cellulose chromatography and gel electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. The presence of disulfide bonds was detected by running samples with and without dithiothreitol and observing alterations in the positions of α chains.

Cell Lines used in these studies: Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

L-929- a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3- a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson.

BALB-3T6 and 3T12- lines of mouse embryo fibroblasts derived from the same pool of cells as 3T3 but non-contact inhibited and tumorigenic.

Ki-3T3- a line established by Aaronson by transformation of Balb 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Embryonic and adult human diploid fibroblasts-purchased commercially.

Major Findings:

I. Submicrosomal localization of lysyl hydroxylase.

We have confirmed our preliminary observations that only a fraction of lysyl hydroxylase is intracisternal as compared to prolyl hydroxylase which is almost entirely intracisternally localized.

II. The role of ascorbic acid in collagen metabolism.

A. Identity of a reducing cofactor for prolyl hydroxylase in cultured cells.

1. Kirsten sarcoma virus transformed BALB 3T3 cells (Ki-3T3).

These cells exhibit 60-90% of maximum proline hydroxylation in the absence of ascorbate (ascorbate-independent proline hydroxylation). We used an assay similar to that for prolyl hydroxylase to measure reducing activity in cell extracts. All components except the reducing compound were present in excess and the system responded to reducing substances in a linear fashion. It was found that extracts of Ki-3T3 contained some reducing activity but the activity could be increased by adding NADH. In contrast, extracts of the parent BALB 3T3 cells, which show a much greater requirement for ascorbate for proline hydroxylation than the transformant, had little or no activity unless NADH was added. These results suggest that the reducing substance exists in an oxidized and reduced state which may be regulated in cells by the level of NADH. The amount of cofactor in the reduced state is expressed as reducing activity in the absence of NADH compared to the amount of activity in the presence of NADH. Ki-3T3 cell extracts were found to contain cofactor which was about 30-40% reduced

while 3T3 extracts had about 0-4% reduced. This could explain the difference in the ascorbate requirement of these lines.

Tetrahydropterin concentrations were measured in extracts of both cell lines and although the levels were 8-10 times higher in the extract of the transformant, the concentration was 1/1000 that calculated to be present based on the activity of the extract compared to a dimethyltetrahydropterin standard. This suggests that the reducing substance is not tetrahydropterin. Tetrahydrofolate was also ruled out as a significant contributor to ascorbate-independent proline hydroxylation since aminopterin, which inhibits tetrahydrofolate formation, did not reduce proline hydroxylation in the absence of ascorbate.

Fractionation of Ki-3T3 cell sonicates by differential centrifugation revealed that most of the reducing substance was present in particles which are probably microsomes, although we have not conclusively characterized them.

2. L-929 cells.

This cell line also exhibits ascorbate-independent proline hydroxylation but only in the stationary phase of growth. In log phase, there is a requirement for ascorbate in order to obtain maximum hydroxylation. Reducing substance was detected in extracts of stationary L-929 cells but little or none was found in log phase extracts. When NADH was present during the assay, however, activity increased in log extracts but there was no effect on the activity in stationary extracts. In addition, the total amount of oxidized and reduced substance (activity in the presence of NADH) was 2-4 times greater in stationary extracts than in log extracts.

The results of these experiments support our previous proposals that ascorbate-independent proline hydroxylation is due to a reducing substance other than ascorbate which is present in greater amounts in Ki-3T3 compared to 3T3 and in stationary compared to log phase L-929 cells.

B. Mechanism of action of ascorbic acid.

We previously found that ascorbate stimulates collagen secretion because of its role as a cofactor for proline hydroxylation. We are now carrying out experiments to determine whether this compound plays an additional role in regulating collagen synthesis and/or degradation by pulse-chase types of experiments.

III. Effect of dbcAMP on transformed 3T3 cells.

A. Collagen phenotype of normal and transformed cells.

Four distinct genetic types of collagen are known at present. Type I found in almost all tissues but uniquely in calcified bone and tendon, is composed of 2-α_1 type I chains and 1-α_2 chain: $[\alpha_1(I)]_2\alpha_2$. Type II col-

lagen, $[\alpha\ (II)]_3$, is found in cartilage; type III, $[\alpha_1(III)]_3$, mainly in embryonic tissue and adult blood vessel walls and distinguished from other collagen types by having intrahelical S-S bonds; and type IV in basement membrane. We previously found that both Ki-3T3 and 3T3 cell cultures contained mainly type I collagen but that, in addition, 3T3 contained a small amount of type X while Ki-3T3 contained no type X but a small amount (25-30%) of type Y which appears to be similar to type III collagen. We have analyzed the collagen from several different contact-inhibited clones of 3T3 as well as several types of transformants in order to determine whether the Ki-3T3 phenotype is characteristic of transformed cells. It was found that all contact-inhibited clones contain types I and X while the RNA sarcoma virus transformed cells contain I and Y. The transformants included another isolate of Kirsten sarcoma virus transformed 3T3 and a Moloney sarcoma virus transformant. Cells transformed by the DNA virus SV-40 exhibited exactly the same phenotype as the contact-inhibited cells. A line transformed by 4-nitroquinoline-1-oxide contained collagen different from that of any of the other lines. These results suggest that the collagen phenotype can be altered by transformation and the change is specific for the transforming agent. In addition, the appearance of type Y collagen is one of the few transformed properties which differs in DNA and RNA virally transformed cells, suggesting that there is some difference in the mechanism of transformation by these two types of viruses.

Proposed Course of Research:

I. Localization of lysyl hydroxylase.

Experiments will be carried out to determine how the non-intracisternal enzyme is bound to the microsomal membrane.

II. Role of ascorbate in collagen metabolism.

A. Reducing cofactor.

Purification and characterization of this particulate substance will be carried out and the mechanism of its regulation in cells will be studied.

B. Mechanism of action of ascorbate as Vitamin C.

Studies will continue on the effect of ascorbate on collagen synthesis and degradation.

III. Modification of the collagen phenotype by transformation.

Methods will be devised to make detection of type Y collagen simpler so that additional transformed lines can be screened to determine whether this collagen type specifically appears in sarcoma virus transformants.

Significance to Biomedical Research and the Program of the Institute: Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. In addition, our studies with transformed cells indicate that, at least under some conditions, animal cells may be able to synthesize a substitute for the function of vitamin C in connective tissue.

Our studies with dbcAMP are aimed at determining the extent to which this chemical reverses cell transformation.

Publications:

Evans, C., and Peterkofsky, B.: Ascorbate-independent proline hydroxylation resulting from viral transformation of BALB 3T3 cells and unaffected by dibutyryl cAMP treatment. J. Cell. Physiol. 89: 355-367, 1976.

Peterkofsky, B., and Assad, R.: Submicrosomal localization of prolyl hydroxylase from chick embryo limb bone. J. Biol. Chem. 251: 4770-4777, 1976.

Peterkofsky, B. and Prather, W.: Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. J. Cell. Physiol. 90: 61-70, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 5214-06 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

DNA Synthesis in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Wilson	Medical Officer	LB NCI
OTHER:	Y.-C. Chen	Visiting Fellow	LB NCI
	S. Planck	Guest Worker	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An improved method for purification of DNA polymerase alpha from mouse myeloma has been developed. The method involves gentle homogenization in an isotonic buffer at pH 7.6 and then centrifugation at 100,000 x g; the supernatant fraction is adjusted to pH 6.0 and chromatographed on CM-sepharose, then DEAE-sepharose, and finally oligo(dT)-cellulose. Alpha-polymerase is obtained in near homogenous form in three days with a recovery of about 20%. The effect of purified myeloma DNA binding proteins upon the activity of the purified alpha-polymerase is under investigation. In addition a protein factor has been identified that stimulates the enzyme as it replicates single-stranded calf thymus DNA. This factor appears distict from the typical DNA binding proteins isolated on DNA-cellulose columns.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: The objective of this research program is to understand mechanisms involved in DNA synthesis in mammalian cells. Our approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

Our previous analysis of the DNA polymerases of mouse myeloma revealed three enzymes that are analogous to DNA polymerases now well known in a variety of mammalian tissues. We have succeeded in purifying two of these enzymes and are involved in rigorous characterization of their molecular and biochemical properties.

We anticipate that these studies, in conjunction with those of other workers, on the properties and specificities of the purified DNA polymerases will help answer important question about mechanisms of DNA synthesis in vivo.

Methods Employed: Procedures have been developed for purification of DNA polymerases from mouse tissue. These procedures involve subcellular fractionation, various types of ion exchange, gel filtration, and affinity chromatography, and assays for DNA polymerase activities using a variety of reaction conditions. Cell fractionation procedures and characterizations of reaction products were performed using conventional techniques of rate-zonal centrifugation and scintillation spectrophotometry.

Major Findings:

1. An improved method for purification of DNA polymerase alpha:

A new method for purification of DNA polymerase alpha has been developed. This new method takes advantage of the special affinity of the DNA polymerase for poly(dT).

We had found previously that alpha-polymerase activity in extracts from myeloma MOPC-104E is not associated with a single protein molecule, but with several molecular species that differ in isoelectric point. Since these different species could be the result of different extents of proteolytic cleavage of the same enzyme molecule, the new purification procedure is designed to minimize the activity of proteolytic enzyme. These "anti-protease" measures include homogenation at pH 7.6 in an isotonic buffer such that lysozomes remain intact, inclusion of the serine protease inhibitor, phenylmethylsulfonyl fluoride, throughout the procedure, and separation of the enzyme from lysozomes in the first step. Protein in a 100,00 x g supernatant fraction containing about 80% of the total enzyme activity is chromatographed at pH 6.0, first on a CM-Sepharose column, and then on a DEAE-Sepharose column using ammonium sulfate for the elutions. Finally, the enzyme containing fraction is passed through an activated DNA-cellulose column at pH 7.6, and the flow through fractions are chromatographed on oligo(dT)-cellulose at the same pH. Alpha-polymerase is thus obtained in 3 days at near homogeneity. The recovery of total enzyme activity is about 20%, a much higher value than those pre-

viously reported for preparation of highly purified enzyme, and the yield of enzyme protein is about 0.15 μg/gram of tissue.

Analysis of the polypeptide content of the purified enzyme by SDS-polyacrylamide gel electrophoresis reveals three peptides accounting for most of the visible dye stained material in the gels. Their apparent molecular weights are approximately 235,000, 176,000 and 51,000. Rigorous definition of the subunit composition of this enzyme requires more work, however we have developed a working model on the basis these electrophoresis results: since the molecular weight of the native enzyme is about 230,000, we believe the enzyme is one polypeptide chain of approximately 235,000 daltons; the native enzyme is unusually susceptible to cleavage at a site resulting in two chains (176,000 and 51,000 daltons) without loss of DNA polymerase activity.

2. Identification of a factor required by the alpha-polymerase for replication of single-stranded DNA:

The purified myeloma α-polymerase is unable to replicate long strands of single-stranded DNA, although it is fully capable of initiating new DNA chains on an appropriate primer. This finding, directly analogous to findings with E. coli DNA polymerase II, suggested that our "pure" enzyme had been separated from a factor required for the replication of single-stranded DNA. Thus, using an artificial template-primer containing a phased initiation site directly preceding a long stretch of single-stranded DNA template, we screened tissue extracts and located a factor that enables the polymerase to replicate the single-stranded template. This factor has now been purified through three steps of ion exchange column chromatography. It is completely free of DNA polymerase activity and is active at a very low protein to template ratio, suggesting that it is not a typical DNA binding protein as described below.

3. Purification of myeloma DNA binding proteins:

Using a modification of the procedure described by Herrick and Alberts (J. Biol. Chem. 251: 2124, 1976) have isolated DNA binding proteins from myeloma. These proteins are not retained on a column of double-stranded DNA-cellulose, but are retained on single-stranded DNA-cellulose and remain bound during a rinse with 0.5 mg/ml dextran sulfate or 0.1 M NaCl; the proteins are removed from the column by 2 M NaCl. Analysis on SDS-polyacrylamide gels reveals that these single-stranded DNA binding proteins are a mixture of 10-15 individual polypeptides. As in calf thymus, this group of proteins is abundant in myeloma, comprising about 1% of total cell protein. We have found that the mixture can be resolved into individual polypeptides by gradient elution from single-stranded DNA cellulose followed by isoelectric focusing on in polyacrylamide gels. The individual DNA binding proteins are being being characterized with regard to amino acid content, DNA unwinding ability, and effect on the activity of myeloma DNA polymerases.

Significance to Cancer Research: Detail knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing enzymes is necessary for maintenance of a rapid rate of cell division, and in some cases these enzymes may play an important role in the development of the neoplastic state.

Proposed Course of Research: The objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells; the main approach is the investigation of the mechanism of action DNA synthesizing enzymes *in vitro*.

1. To purify and characterize the DNA polymerases alhpa and gamma of mouse myeloma.

2. To investigate the mechanism of action of purified DNA polymerase alpha using the prototype system of poly(dT) as template and oligo(rA) as primer.

Publications:

Kuff, E.L., Lueders, K.K., Orenstein, J., and Wilson, S.H.: • Differential response of type-C and intracisternal type-A particle markers in cells treated with Iododeoxyuridine and Dexamethasone. J. of Virology 19: 709-716, 1976.

Matsukage, A., Sivarajan, M., and Wilson, S.H.: Studies on DNA alpha-polyumerase of mouse myeloma: Partial purification and comparison of three molecular forms of the enzyme. Biochemistry 15: 5305-5314, 1976.

Pitha, J., and Wilsom, S.H.: A template specific inhibitor of mammalian DNA polymerases. Nucleic Acids Res. 3: 825-834, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 5215-06 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biological and ultrastructural studies on cell cooperation in the immune response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Emma Shelton Research Biologist LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the effect of various inhibitors and of temperature on surface topology of mouse lymphocytes macrophages and mast cells. The mast cell surface proved very resistant to effects of these agents. Inhibitors of energy metabolism are promoters of microvillus extension on lymphocytes and macrophages. Local anaesthetics and glutathione oxidizing agents (viz. diamide) are inhibitors of microvillus extension. Low temperature affects surface topology and over-rides the effect of antimetabolites. Low temperature and diamide reciprocally compete; in the presence of both, the cell surface appears normal. These promoters and inhibitors provide a way to examine the control of cell surface topology.

Double fixation of cells in glutaraldehyde and osmium tetroxide induces blebs in cell membranes. No remedy for this artifact has been discovered.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To examine the interaction of lymphocytes and macrophages co-operating in the immune response; specifically, to examine the factors controlling the surface topology of these cells.

Methods and Major Findings:

1. Effect of various inhibitors and temperature on surface topology.

Lymphocytes, macrophages and mast cells rinsed from the unstimulated peritoneal cavity of mice and allowed to settle on substrates are prepared for scanning electron microscopy (SEM). When incubated at 37°C for 20 minutes or less, the lymphocyte surface is covered with irregularly spaced, short microvilli, the macrophage surface is irregularly folded, while the mast cell surface has sharply defined folds and stubby microvilli.

It is now known that microtubules and microfilaments (actin and myosin) to a large extent control cell shape and cell locomotion. In an attempt to understand the factors that control the surface topology of these cells we have examined the effects of (1) temperature, (2) inhibitors of energy metabolism, sodium azide, sodium cyanide, di-nitrophenol, (3) a compound binding tubulin, colchicine, (4) a microfilament "disrupter", cytochalasin B, (5) glutathione oxidizing agents, diamide and iodoacetamide, and (6) local anaesthetics, dibucaine and lidocaine.

Colchicine and cytochalasin B produce over-all alterations in cell shape, but do not markedly affect lymphocyte microvilli. Macrophage surface topology and shape are changed by cytochalasin B.

Inhibitors of energy metabolism have a profound and surprising effect upon surface microvilli. They are promoters of microvillus elongation. In a timed sequence after exposure to 10 nM sodium azide at 37°C, first macrophages then lymphocytes became completely and uniformly covered with long microvilli. Mast cells are resistant to this effect. The effect is reversible by washing in buffered saline.

In contrast to this effect, local anaesthetics and glutathione-oxidizing agents are inhibitors of microvillus formation. Exposure to 0.5 mM diamide at 37°C causes macrophages and lymphocytes and ultimately mast cells to acquire a completely smooth surface. The effect is reversible within 5 minutes by exposure to di-thio-erythreitol. Similar effects of dibucaine and lidocaine are reversible, but those of iodoacetamide are not. Azide acting as a promoter and diamide acting as an inhibitor of microvillus formation provide a way to study the cellular control of surface topology.

Low temperature also reversibly affects the cell surface toplogy and inhibits and over-rides the effect of anti-metabolites. At 2°C, sparse, long, irregularly shaped microvilli and lamellopodia project from the lymphocyte surface, the macrophage surface is deeply folded while the

mast cell surface is little affected. Upon reducing the temperature from 37°C to 2°C in graded stages, the effect begins to appear at 15°C and becomes progressively more marked as 2°C is approached. It does not seem to be time dependent between 15 and 45 minutes. These features charácteristic of low temperature, are not altered during a 20 minute exposure to 10 mM sodium azide. Temperature and diamide reciprocally modify the effect of each other upon the cell surface, thus, after 20 minutes at 2°C in the presence of 1 nM diamide the cells maintain the features of untreated cells.

2. Blebs: an artifact of "double" fixation in glutaraldehyde and osmium tetroxide: --a hazard for transmission electron microscopy.

In the experiments described above, cells were examined in the SEM. Transmission electron microscopy, TEM, must be used to permit a view beneath the cell membrane. For SEM, cells are fixed in glutaraldehyde alone, but for TEM, such fixation must be followed by OsO_4 fixation or membranes cannot be seen. It was discoverd that such "double" fixation induces membrane blebbing in peritoneal cells, cultured cells of various types and tissues fixed in situ. Peritoneal cells are particularly sensitive. Fixation in glutaraldehyde or osmium tetroxide alone does not induce blebbing. The blebbing is not prevented by variations in time of fixation, temperature of fixation, osmolarity or reagents, concentration of reagents, by di-valent cations, by chromium or by a variety of variations of protocol such as rinsing or omitting a rinse in buffer or use of different buffers. The blebbing is prevented in peritoneal cells by adding 1% tannic acid to the glutaraldehyde in the initial fixation, but it does not prevent blebbing in cultured cells or in cells in situ. Furthermore, such fixation results in broken membranes and poor fixation as judged by examination in TEM. In TEM, the blebs themselves appear as empty blisters bounded by a membrane bi-layer.

The effect of double fixation is interpreted as the creation of weak spots in the membrane lipid bi-layer by the cross-linking of proteins by the glutaraldehyde and the subsequent "blow-out" of the lipid bi-layer after exposure to osmium tetroxide, a compound that is known the react with lipids.

We are confronted with an artifact that may jeopardize our ability to interpret events occurring at the membrane. We have no solution to the problem at the moment.

Proposed course: To analyze the effect of temperature, azide and diamide upon surface topology of peritoneal cells.

Publications:

Orenstein, J.M., Johnson, L., Shelton, E., and Lazzarini, R.A.: The shape of vesicular stomatitis virus. Virology 71: 291-301, 1976.

Orenstein, J.M., and Shelton, E.: Surface topography and interactions between mouse peritoneal cells allowed to settle on an artificial substrate: Observations by scanning electron microscopy. Exp. Mol. Path. 24, 201-219, 1976.

Shelton, E., Bennett, W.I., and Orenstein, J.M.: Differentiation of small numbers of mouse peritoneal cells into antibody-producing plasma cells in diffusion chambers. Exp. Mol. Path. 24: 220-235, 1976.

Orenstein, J.M., and Shelton, E.: Surface topography of leukocytes in situ: Cells of mouse peritoneal milky spots. Exp. Mol. Path. 24: 415-423, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 5241-02 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Transcription of covalently linked cellular and SV40 viral DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Kuff	Head, Biosynthesis Section	LB NCI
OTHER:	G. Khoury	Head, Section on Virus Tumor Biology	LDNATV NCI
	F. J. Ferdinand	Visiting Fellow	LDNATV NCI

COOPERATING UNITS (if any)

Laboratory of DNA Tumor Virus
NCI

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are examining the transcription of covalently linked cellular and viral DNA sequences during infection with host substituted variants of SV 40.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To use the host-substituted variants of SV40 in studying the transcription of viral DNA and the cellular processing of transcripts containing covalently linked host and viral RNA sequences.

Methods Employed: Cell culture; virus passage; preparation of viral transcriptional complexes; nucleic acid labeling; isolation and hybridization; other virological, biochemical and molecular biological techniques.

Results: Viral transcriptional complexes were extracted from the nuclei of monkey kidney cells infected with wild type SV40 or a variant strain containing a high proportion of host-substituted DNA molecules. The RNAs synthesized by these complexes in an *in vitro* system were analyzed for their content of SV40 and host sequences by a technique of sequential hybridization to plaque purified and substituted viral DNAs. The relative labeling of the two types of sequences was commensurate with their proportion in the viral DNA (about 20% host). The substituted virus contains both reiterated and unique types of cellular sequences, and both kinds were transcribed. Transcripts of the substituted sequences formed a very much smaller proportion of the virus-related RNA recovered from intact infected cells, suggesting either that host sequence transcripts are synthesized but rapidly degraded in the whole cell, or that transcription of these sequences are artificially enhanced in the *in vitro* system. We compared the self-annealing of viral RNAs from nuclear extracts of cells infected with wild type and substituted virus; transcripts labeled both *in vivo* and *in vitro* showed a 2 to 3-fold higher level of self-annealing in the case of the variant.

Proposed Course of Research: Future studies will employ cloned substituted SV40 variants. Host DNA sequences will be excised with appropriate restriction endonucleases and immobilized by attachment to insoluble substrates. Early events in transcription and RNA processing will be studied in detail.

Publications:

Oren, M., Kuff, E.L., and Winocour, E.: The presence of common host sequences in different populations of substituted SV40 DNA. *Virology* 73: 419-430, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 3212-02 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Cell Nutrition, Growth and Neoplastic Transformation In Vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. M. Price	Research Biologist	LB NCI
Other:	K. K. Sanford	Head, Cell Physiology & Oncogenesis Section	LB NCI
	R. R. Gantt	Research Biologist	LB NCI
	W. G. Taylor	Research Biologist	LB NCI
	C. U. Garrison	Lab. Technician	LB NCI

COOPERATING UNITS (if any) R. Parshad, Department of Pathology, Howard University, Washington, D.C.; R. Merwin, Viral Leukemia and Lymphoma Branch, NCI; J. Selkirk, Oakridge National Laboratory, Oakridge, Tennessee; R. Deely, Cellular Regulation Section, LB, NCI.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine the in vitro nutritional requirements of freshly isolated cells and long-term lines, both neoplastic and nonneoplastic and to improve culture conditions of the cells so that rigidly controlled cells can be used to study the differences between nonneoplastic neoplastic or malignant cells at nutritional, metabolic, karyologic and morphologic levels.

PHS-6040
(Rev. 10-76)

Project Description:

A program to improve culture conditions so that rigidly controlled cells can be used to study differences between nonneoplastic and neoplastic cells at nutritional, metabolic, karyologic and morphologic levels.

Objectives:

1. To determine the effect of horse and fetal bovine serum and 1% and 18% oxygen on chromosome stability, presence of fetal antigen and DNA breakage of C57Bl mouse lung cells.

2. To determine if a more sensitive in vivo assay for neoplastic transformation can be obtained by implanting glass helices carrying mouse cells into syngeneic hosts.

3. To develop techniques for culturing normal human epithelial cells and to attempt to transform these cells by chemical carcinogens and study differences between normal and neoplastic human epithelial cells at nutritional, metabolic, karyologic and morphologic levels.

4. To develop methods for culturing chick liver cells in vitro. Once in culture those cells will be used to study regulation of certain metabolic functions of chick liver.

Methods Employed: Cell cultures derived from adult C57Bl mouse lung were cultured in medium containing either horse serum or fetal bovine serum and cultures grown in both sera were gassed with 1% and 18% oxygen-containing gas. The cells were tested at various intervals for presence of fetal antigen, changes in chromosomes and for neoplastic transformation.

Techniques were devised to culture normal human epithelial cells. Primary cultures of human epithelial cells were treated with various chemical carcinogens to attempt to induce neoplastic transformation.

Methods were developed for culturing chick liver cells in vitro.

Major Findings:

1. The effect of horse and fetal bovine serum and 1% and 18% oxygen on chromosome stability, DNA breakage, production of fetal antigen and neoplastic transformation in C57Bl mouse lung cultures. The chromosome number of C57Bl mouse lung cells remained more stable when the cells were grown in an atmosphere containing 1% oxygen than when the cells were cultured in the presence of 18% oxygen. This effect was noted regardless of the serum which was in the culture medium.

All of the cell lines tested showed no presence of fetal antigen through 10 weeks in vitro. After approximately 20 weeks in culture the cells grown in fetal bovine serum and 1% oxygen showed some fetal antigen. After 40 weeks

in vitro the cells in norse serum and 1% oxygen showed fetal antigen as well as those cells in fetal bovine serum in either oxygen concentrations. There appeared to be more fetal antigen present, however in the cells grown in fetal bovine serum in 1% oxygen.

All cell lines tested became neoplastic by in vivo assays. The latent

period for cells grown in medium containing horse serum was shorter (2 months) than for all other lines tested (from 6 to 8 months).

2. Development of a more sensitive assay system for neoplastic transformation. C57Bl mouse cells were grown on the surface of 3 mm glass helices placed in a Yoshi culture dish. Under the direction of Dr. Ruth Merwin a small number of the helices carrying cells were implanted subsutaneously into syngeneic hosts. From 3 to 6 helices were placed in each animal. Each helix contained approximately 100 cells. Tumors were obtained in 3 of 4 cell lines injected indicating that it is not necessary to have very large numbers of cells. The latent period for the tumors was slightly longer than for tumors derived by the usual method of inoculating at least 10^6 cells intramuscularly.

3. Development of techniques for the cultivation of human epithelial cells and treatment of those cells with chemical carcinogens.

Human epitnelial cultures are obtained by placing 1-2 mm explants into culture flasks coated with serum. Within 7-10 days epithelial growth is obtained and the explants are removed. Persistant fibroblasts are eliminated by frequently trypsinizing the culture. The epithelial cells remain attached. All attempts at subculturing the epithelial cells have failed. We are now in the process of trying to grow the epithelial cells on X-irradiated feeder layers of fibroblasts in the hope of attaining better growth and perhaps the ability to subculture the cells.

We are also trying to improve the growth medium and thus obtain faster growth as well as the ability to subculture the epithelial cells. Early studies have indicated that better growth of these cells is obtained using a 50:50 mixture of NCTC 135 and NCTC 163. Apparently the NCTC 135 medium is contributing needed vitamins, coenzymes, etc. NCTC 163 contains fewer components but has larger amounts of amino acids and some vitamins. Further work is being carried out to determine exact chemicals and the amounts needed for optimum growth of human epithelial cells.

Attempts to neoplastically transform epithelial cells with benzo (a) pyrene (BP) have been unsucessful although we have determined that human epithelial cells metabolize this carcinogen. We have learned that 1 microgrom/ml BP is cytotoxic and kills around 50-75% of the cells in a given culture. Cells 50 treated are being maintained in culture and are being given 0.1 microgram/ml tetradecanoly phorbol. acetate to aid in transformation.

4. Development of methods for the cultivation of chick liver cells.

Initial attempts at culturing chick liver cells were made with young roosters 3 to 9 weeks old. We tried several media including NCTC 135 and NCTC 163 and Leibovitz L.15. The NCTC 163 and L.15 both contain large amounts of amino acids which we thought would be helpful. It appears thus far that better growth is obtained with NCTC 135. Fetal bovine serum is used as a supplement. We have found that more success is obtained by dispersing the liver tissue with pronase than by just mincing and planting liver explants.

Significance and Proposed Course:

1. It appears from this study that mouse cells grown in an atmosphere of 1% oxygen have more chromosome stability than those in 18% oxygen. Fetal antigens were found to be retained even after 5 years in cell lines derived from fetal tissue. In these lines no consistent qualitative or quantitative change in fetal antigen expression could be correlated with neoplastic transformation. These studies have been prepared for publication. We are continuing to study the effect of oxygen concentration on neoplastic transformation *in vitro*.

2. This was a short-term project to determine whether assay methods requiring fewer cells could be used for *in vivo* assay for neoplastic transformation. This project is now terminated. However the techniques employed may be useful later for assaying cell lines which do not proliferate rapidly or do not readily become established in long-term culture such as human epithelial cultures.

3. Most human cancers are carcinomas. It would be beneficial if *in vitro* studies can be carried out with epithelial cells rather than fibroblasts. We are continuing our efforts to find better techniques for culturing human epithelial cells. We shall also continue to try to neoplastically transform the human epithelial cultures attained with various chemical carcinogens. Studies are also underway to determine the effect of light on human epithelial cultures and also the interaction of light on chemical carcinogens. Other studies to be carried out involve the use of certain tumor viruses to attempt to induce carcinogenesis of human epithelial cells.

4. This project has been terminated.

Publications:

1. Price, F.M. and Sanford, K.K.: Cleaning and preparation of glassware for cell culture. Tissue Culture Association Manual 2: 379-382, 1976.

2. Parshad, R., Sanford, K.K., Jones, G.M., Price, F.M. and Taylor, W.B.: Oxygen and light effects on chromosomal aberrations in mouse cells *in vitro*. *Exp. Cell Res.* 104: 199-205, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 3213-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Physiology and Carcinogenesis of Mammalian Cells in Culture.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. K. Sanford	Head, Cell Physiology & Oncogenesis	LB NCI
Other:	R. R. Gantt	Research Chemist	LB NCI
	S. Handleman	Biologist	LB NCI
	G. Jones	Microbiologist	LB NCI
	C. Garrison	Biological lab. Technician	LB NCI
	A. Kerr	Phys. Sci. Technician	LB NCI

COOPERATING UNITS (if any) 1. R. Parshad, Department of Pathology, Howard University, School of Medicine, Wash. D.C. 2. R. E. Tarone, Biometry Branch, NCI. 3. B. Wetzel, Dermatology Branch, NCI.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.35	1.75	1.60

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of four studies with the following objectives:

1. To define those culture conditions that induce or prevent chromosomal instability, DNA damage and "spontaneous" neoplastic transformation of rodent cells in culture as compared with human cells.
2. To establish whether the topography of neoplastic and nonneoplastic cells in culture differs and is influenced by cell cycle.
3. To analyze and explain differences in locomotion of nonneoplastic and neoplastic cells of common origin.
4. To develop and evaluate rapid tests for neoplastic transformation in vitro.

PHS-6040
(Rev. 10-76)

Project Description:

This project consists of the following studies:

Study 1. Effect of fluorescent light and oxygen on chromosomes, DNA, and neoplastic transformation of mouse cells in culture.

Objective: To define those culture conditions that induce or prevent chromosomal instability and DNA damage in cultured cells; to analyze the mechanisms; and to evaluate this type of genetic change as a causative factor in "spontaneous" neoplastic transformation of rodent cells as compared with human cells in culture.

Major Findings:

1. In studies with Dr. Gantt, the DNA of mouse cell cultures exposed to fluorescent light (150 foot-candles, 20 hours) eluted more slowly in the Kohn alkaline elution assay than that from the shielded controls. This slower elution is interpreted to mean the DNA is crosslinked in some way with protein and/or DNA because the elution rate has been shown by Kohn and coworkers to be related to the molecular weight; i.e., the larger the molecular weight the slower the elution.

2. In studies with Dr. Parshad, visible light and oxygen were found to cause chromatin breaks in rodent cells. Experiments to date indicate that the effective wavelength for both DNA crosslinking and chromatin breaks lies between 400 and 450 nm and is probably one of the mercury emission lines at 405 or 436 nm.

3. The addition of catalase or glutathione to the culture medium during light exposure significantly reduces or abolishes the incidence of light-induced chromosome aberrations.

4. In limited studies to date, repeated (2 or 3x weekly) exposure of mouse cells to fluorescent light (150 foot-candles, 3 or 20 hours) produces or accelerates neoplastic transformation as compared with cultures shielded from light during routine handling.

5. Exposure of human diploid fibroblasts to fluorescent light (3-4 hours, 1-3x weekly) reproducibly enhances the proliferation rate and extends their lifespan in culture. Single or multiple exposures of short duration stimulate proliferation, whereas exposures of long duration were cytotoxic. This proliferative response is reversible and mediated through the culture medium.

Proposed Course: This study will be continued with emphasis on similarities and differences in the responses of rodent and human cells to light and oxygen, DNA repair, and relationship with neoplastic transformation. Studies are in progress to identify the growth-stimulating substance(s) produced in the medium by visible light.

Significance: Rodent cells in culture are known to undergo both chromosomal aberrations and "spontaneous" malignant transformation. In contrast, human cells in culture are difficult to transform and their chromosomes are far more stable. The present results suggest that production of H_2O_2 and a high redox potential of the medium are causative factors in the karyotypic instability of mouse cells and that karyotypic instability and/or DNA damage produced by singlet oxygen may be a causative factor in neoplastic transformation of mouse cells in culture. Although visible light produces DNA cross-linking in human fibroblasts, further studies are in progress on repair of this damage and karyotypic stability of human as compared with mouse cells.

The production of a substance in the culture medium by visible light that influences both proliferation rate and lifespan of human diploid cells, when identified, should be an important contribution to the nutrition of these cells in culture.

Study 2. The surface topography of paired neoplastic and nonneoplastic cells in culture and the influence of cell cycle on surface features (collaboration with Dr. Wetzel).

Objectives:

1. To compare the surface features (microvilli, blebs, and ruffles) and form of paired neoplastic and nonneoplastic cells to establish whether the less-spread neoplastic cell has excessive amounts of membrane stored in cell projections and appears to have a greater surface activity.

2. To establish whether the cell cycle largely determines the topography of cells in culture.

Major Findings: The results reported in last year's annual report concerning comparison of topography of neoplastic and nonneoplastic cell lines have been publisned. By combined autoradiography, time lapse, and scanning microscopy, data were obtained that challenge the popular concept that cell cycle largely determines the topograpnic features of cells in culture. Cells of the CHO-K1 line were used. The results with a limited number of cultures indicated that the cell cycle exerts little influence on the topography of interphase cells in asynchronous culture.

Proposed Course: This study will be continued to obtain more complete data. A survey and classification of all cells in randomized samples of the culture will be carried out for stage in the cell cycle and surface morphology.

Significance: Cell surface features such as microvilli, blebs, and ruffles are cell organelles little understood and reputed to vary with neoplastic state and cell cycle. The present studies help to clarify these relationships.

Study 3. Locomotor behavior of nonneoplastic and neoplastic cells in culture (Collaboration with Dr. Tarone)

Objective: To analyze and explain differences in locomotive pattern in nonneoplastic and neoplastic cells of common origin.

Major Findings: Cells in all cultures of nonneoplastic lines exhibited a "persistent" walk when measured over short 2.5 hour intervals, whereas cells from the neoplastic lines had a random pattern of locomotion. No relationship between cell density and randomness of locomotion was observed, and the nonneoplastic cells appeared to proliferate as rapidly as the neoplastic. However, the neoplastic cells had higher rates of locomotion.

Significance: This observation concerning the random pattern of locomotion indicates a certain loss of polarity in the neoplastic cell. The organ of locomotion in a fibroblast is the leading edge, a broad protrusion of lamellar cytoplasm with attachments to substrate for traction. The deficient amount of lamellar cytoplasm in the neoplastic cell with possibly fewer attachment sites and the higher migration rate may account for the loss of a persistent walk observed in this study. This change in locomotion may possibly be associated with invasive potential of the cells in vivo.

Proposed Course: This study has been submitted for publication and will be terminated.

Study 4. Colony morophology and growth in agarose as tests for spontaneous neoplastic transformation in culture.

Objectives:

1. To develop and evaluate rapid tests for spontaneously transformed neoplastic cells in culture.

2. To establish whether spontaneously transformed cells exhibit properties similar to virus-transformed cells.

Major Findings: This study was completed and has been published.

Honors, Awards and other Activities:

1. Member, Executive Committee, Tissue Culture Association.
2. Member, Editorial Advisory Board of Cancer Research.
3. Member, Terminology committee, Tissue Culture Association.
4. Member, Program Committee and Executive Board, 3rd Decennial Review Conference on "Gene Expression and Regulation in Cultured Cells".

Publications:

1. Sanford, K.K., Hall, W.T., Hobbs, B.A., and Hursey, M.L.: Ruthenium red-staining coat of paired neoplastic and nonneoplastic mouse cell lines in culture. J. Natl. Cancer Inst. 56: 1031-1033, 1976.

2. Taylor, W.G., Evans, V.J., Fox, C.H., Camalier, R.F., and Sanford, K.K.: Evaluation of the dyna-cell vessel for bulk production of substrate-dependent cells. Biotechnol. Bioeng. 17: 1847-1851, 1975

3. Sanford, K.K., Handleman, S.L., and Jones, G.M.: Morphology and serum dependence of cloned cell lines undergoing spontaneous malignant transformation in culture. Cancer Res. 37: 821-830, 1977.

4. Parshad, R. and Sanford, K.K.: Oxygen and light effects on chromosomal aberrations in mouse cells in vitro. Exper. Cell Res. 104: 199-205, 1977.

5. Price, F.M. and Sanford, K.K.: Cleaning and preparation of glassware for cell culture. Tissue Culture Association Manual 2: 379-382, 1976.

6. Wetzel, B., Jones, G., and Sanford, K.K.: Cell cycle and topography in non-synchronized monolayers: the use of autoradiography and time-lapse for more rigorous SEM studies. Scanning Electron Microscopy 1: 545-552, 1977.

7. Wetzel, B., Sanford, K.K., Fox, C.H., Jones, G.M., Westbrook, E.W., and Tarone, R.E.: Topography of nonneoplastic and neoplastic cells of common origin. Cancer Res. 37: 831-842, 1977.

8. Fox, C.H., Caspersson, T., Kudynowski, J., Sanford, K.K., and Tarone, R.E.: Morphometric analysis of neoplastic transformation in rodent fibroblast cell lines. Cancer Res. 37: 892-897, 1977.

9. Tucker, R.W., Sanford, K.K., Handleman, S.L., and Jones, G.M.: Colony morphology and growth in agarose as tests for spontaneous neoplastic transformation in vitro. Cancer Res. 37: 1571-1579, 1977.

10. Parshad, R., and Sanford, K.K.: Proliferative response of human diploid fibroblasts to intermittent light exposure. J. Cell. Physiology, in press.

11. Handleman, S.L., Sanford, K.K., Tarone, R. E., and Parshad, R.: The cytology of spontaneous neoplastic transformation in culture. In Vitro, in press.

12. Proceedings of the Third Decennial Review Conference on Cell, Tissue and Organ Culture. Gene Expression and Regulation in Cultured Cells. Ed. K.K. Sanford. NCI Monograph, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CBD 3234A-04 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Primary Biochemical Alterations Leading to Neoplastic Conversion.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. R. Gantt	Research Chemist	LB NCI
Other:	K. K. Sanford	Head, Cell Physiology & Oncogenesis Section	LB NCI
	A. B. Chepelinski	Visiting Scientist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program is designed to elucidate the primary chemical alterations leading to neoplastic transformation. Various biochemical techniques are being applied to a system developed in the Cell Physiology & Oncogenesis Section, with these immediate objectives in view:

1. To identify the primary changes in nucleic acid methylation during neoplastic transformation and cell regulation.

2. To assess the role of nucleic acid methylation in the RNA tumor virus life cycle and its relationship to transformation.

PHS-6040
(Rev. 10-76)

Project Description:

To elucidate the primary chemical alterations which are necessary biochemical changes leading to neoplastic transformation and to devise methods to detect and control the transformation based on these chemical alterations.

Objectives: To identify primary changes in the methylation of cellular and viral nucleic acids during neoplastic transformation and to exploit these changes to develop an assay for, and possible control of, neoplastic transformation in vitro.

Methods Employed: In addition to the usual laboratory techniques and innovations, we use cloned, paired cell lines to control the wide variations among cell lines and even in the same cell line at different culture generations. These paired lines are described by Dr. Katherine Sanford, Project #3213-02.

Major Findings:

1. The locus of 5-methylcytosine, a ubiquitous minor base in DNA, is randomly distributed along the DNA chain with respect to the nucleosome. The hypothesis that nucleosome binding in eukaryotic DNA is determined by the locus of 5-methylcytosine was tested by isolating nuclei of chicken red blood cells, treatment of the nuclei with S-1 nuclease, isolation of the DNA bound to the nucleosome monomer and the solubilized DNA. Analysis of these DNA fractions showed 5-methylcytosine to be distributed equally between them.

2. The intracysternal "A" type particle, which is a budding, nucleic acid containing, virus-like particle, contains several RNA methylating enzymes. This was shown by rigorous particle purification and sedimentation in sucrose gradients after treatment of the purified particles with 1% triton X-100. The RNA bases modified included adenine, guanine and cytosine.

Significance: The importance of nucleic acid methylation has recently been emphasized by the discovery of post transcriptional on RNA modifications which are required for translation of the message. The presence of at least three RNA methylating enzymes in the core of the intracisternal "A" particles suggests additional modes of post transcriptional regulation of processess involving nucleic acids.

Proposed Course of Investigation:

1. Establish the biological function of the methylase present in the "A" particle cores.

2. Produce and purify antibodies to 5-methylcytosine along the DNA chain by electron microscopy.

3. Establish the content of 5-methylcytosine in rat mitochondnial DNA.

Publications:

1. Gantt, R.R., and Smith G.H.: RNA methylase activity in virus-infected, normal, and neoplastic mouse mammary tissue. In "The Biochemistry of S-Adenosylmethionine", edited by E. Borek, F. Salvatore, F. Schlenk, G. Williams-Ashman, and V. Zappia. Columbia University Press, N.Y. 1976-77,

2. Izzo, P., and Gantt, R.: Partial purification and characterization of N^2-guanine RNA methyltransferese from chicken embryos. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CBD 3257-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Growth and Neoplastic Transformation of Mammalian Cells In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Taylor	Research Biologist	LB NCI
Other:	K.K. Sanford	Head, Cell Physiology & Oncogenesis	LB NCI
	R.R. Gantt	Research Biologist	LB NCI
	F.M. Price	Research Biologist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this program is to design a better synthetic milieu in which freshly explanted or long term mammalian cells will survive and grow. Topics of present interest include: (1) the role of oxygen in survival, growth, and metabolism of cells, particularly sparse cell populations; (2) chemical analyses of a presumed-peptide growth factor for mammalian cells grown in serum-free medium; (3) the growth of human epithelial cells in vitro.

PHS-6040
(Rev. 10-76)

Project Description:

Project No. 1. Enhanced proliferation at physiologic oxygen concentrations.

Objective: Earlier studies showed colony development (i.e., growth) and chromosome stability are enhanced when a gas phase of 1% rather than 18% oxygen is used in conjunction with 10% CO_2. The partial pressure of disvolved oxygen in medium equilibrated with 1% O_2 is comparable to that of blood and tissues (30-40 mm Hg). Studies were designed to determine if: (a) cell populations obtained with a gas phase of 1% O_2 were "density dependent"; (b) the use of 1% O_2 v/v altered glucose utilization and lactic acid production; (c) a lowered P_{O_2} resulted in enhanced attachment and survival, or an increased proliferation rate leading to an apparently greater cell population.

Methods Used: Dissolved gas concentrations and pH were measured with a Radiometer BSM3MK2 blood gas analyzer in conjunction with a PHM 27 meter fitted with a PHA927b gas monitor. Medium glucose concentration was measured with a Beckman glucose analyzer. Lactic acid concentration was measured by a pyridine nucleotide coupled spectrophotometric lactic acid assay. Medium F-12 or Dulbecco-Vogt's modified MEM were used with 10% fetal bovine serum. Cell lines tested included human diploid WI-38 cells, cloned mouse lines NCTC7914 and 7915, and an adult normal human, cell line, NCTC9032, derived from explanted skin. Colonies of greater than 5-10 mm diameter were counted, or total cell populations enumerated with a 6300A Cytograf.

Major Findings: (a) An inverse relationship between colony development and P_{O_2} of the culture medium was observed in the first experiments of this study. Multiple regression analysis of these data confirmed that colony development was significantly effected by the oxygen concentration and initial population size. When, in a similar study, cell numbers (rather than colony counts) were determined, it was clear that with an initial population of 10^4 cells/T-15 or less, 2.5-5 times more cells were obtained with 1% O_2 than in cultures gassed with 18% O_2. (b) Per culture vessel significantly greater concentrations of glucose are used and lactate produced by neoplastic mouse clone NCTC 7915 when 1% O_2 is used. When data are normalized as μmoles/10^4 cells, glucose utilization and lactate production varied inversely with the initial inoculum size and oxygen concentration. A glucolysis ratio of less than 2 was observed in most cases. Studies with "normal" human skin cells showed the greatest glycolytic activity during the first 48 hours, a Pasteur effect was suggested by the data.

Significance and Proposed Course: Whether present as a deliberately introduced CO_2: air mixture or as residual, supernatant gas, a gas phase containing atmospheric oxygen concentrations (18%, medium $P_{O_2} \sim 125$-135 mm Hg.) introduces an essential nutrient at an abnormal concentration. In vivo, inhaled air (P_{O_2} 120-150 mm Hg, depending on altitude) equilibrates with alveolar blood ($P_{O_2} \leq 100$mm Hg), and hemaglobulin maintains a homeostatic

Po_2 and Po_2 . In vitro, a P_{O_2} of 30-40 mm Hg can be obtained by equilibrating the growth medium with 1% O_2: 10% CO_2: N_2 v/v in gas-tight, glass culture vessels for a brief perild. To complete the present study it is important to determine if the enhanced growth at 1% O_2 results from cellular responses to a more physiologic milieu -- that is, does dissolved oxygen with atmospheric oxygen concentration might be caused by production of cytotoxic products such as hydrogen peroxide (Science 191: 468, 1976). Further collaborative studies in the role of dissolved oxygen, light and riboflavin (PNAS 71: 3961, 1974) on chromosome instability are planned.

Project No. 2: Further analyses of a peptide-like growth factor for mammalian cells in serum-free medium.

Objectives: Autoclavable, low molecular weight fractions (PD) from commercially available peptones (tissue digests) promote continuous proliferation of mouse, rabbit, hamster, monkey and human cell lines. Studies included: (a) completion or confirmation of earlier experiments regarding the chemical nature of PD; and (b) preliminary purification of PD by chromatographic procedures.

Methods Used: The bioassay techniques used in these studies has been described (Taylor, W.G. and Evans, V.J., Methods In Cell Biology, Vol. VIII, p.47, 1974). A detailed description of the preparation of PD has been published (Taylor, W.G. and Parshad, R., Methods In Cell Biology, Vol. XV, p.421). CM-52 and gel filtration procedures were assessed for separation of PD into additional fractions.

Major Findings: Preliminary results suggest that fractionation of PD on a P-2 gel filtration system is superior to G-25 gel filtration or with CM-52 ion exchange columns. The biologic effects of these fractions on epithelial cells are being studied.

Significance and Proposed Course: The importance of peptide hormones and particularly fibroblast growth factor (FGF) and epithelial growth factor (EGF), is only now becoming apparent. Contaminating, serum-derived proteases confuse the in vitro assessment of these and similar hormones. Development of nutritionally adequate culture media will facilitate these studies. Furture emphasis includes: (a) assessment of other environmental factors which effect the results these types of studies; and (b) an assessment of PD for the survival and growth of primary cell cultures.

Project No. 3: Procurement and cultivation of human epithelial cells.

Objectives: The objective of these studies was the development of culture systems in which human epithelial cells could by grown reproducibly for in vitro carcinogenesis studies.

Methods: Noncontroversial human tissue is obtained through professional contacts or through research resource avenues within NCI. Cell culture procedures for explanted tissue is described elsewhere (NCI 3212).

Major Findings: Because a reproducible source of "normal" human tissue became available only recently, and because of the difficulity in growing human epithelial cells, the data available is limited at this time.

Significance: Because human tumors are most usually carcinomas and arise from epithelial cell types, and because of the renewed interest in growing human epithelial cells in vitro, the significance of these studies is obvious. The proposed course is to assess the effects of different carcinogens, and particularly on the effects of these carcinogens on cell metabolism.

Project No.4: Effect of intermittent light on chromosome cytology and spontaneous neoplastic transformation. An employee no longer with the Section was involved in this problem (see NCI 3213).

Publications:

1. Taylor, W.G. and Parshad, R.: Peptones as serum substitutes for mammalian cells in culture. Methods in Cell Biology Vol. XV, pp. 421-434, 1977.

2. Parshad, R., Sanford, K.K., Jones, G.M., Price, F.M. and Taylor, W.G.: Oxygen and light effects on chromosome aberrations in mouse cells in vitro Exp. Cell Res. 104: 199-205, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 3264-03 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Spontaneous Neoplastic Change During Growth in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Tucker	Research Associate	LB NCI
Other:	K. K. Sanford	Head, Cell Physiology & Oncogenesis Section	LB NCI
	S. L. Handleman	Biologist	LB NCI
	G. M. Jones	Microbioligist	Lb NCI

COOPERATING UNITS (if any) M. S. Meltzer, Biology Branch, NCI; H. Sato, Biophysical Cytology, Biology Department, University of Pennsylvania, Philadelphia, Pa.; F. Frankel, Microbiology Department, University of Pennsylvania, Philadelphia, Pa., V. Speeg, Laboratory of Molecular Biology, NCI.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5228-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Cytology of Neoplastic Transformation In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. L. Handleman	Biologist	LB NCI
Other:	K. K. Sanford	Head, Cell Physiology & Oncogenesis Section	LB NCI
	G. M. Jones	Microbiologist	LB NCI
	C. U. Garrison	Technician	LB NCI

COOPERATING UNITS (if any)

Dr. Lewis Lipkin, DCBD, NCI
Dr. Robert Tarone, Biometry Branch, NCI

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Laboratory of Biochemistry

SECTION

Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.85	1.25	0.6

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to identify and quantify cytologic characteristics of cells in culture that are associated with their neoplastic transformation by chemical carcinogens as well as for following the progresive often very subtle changes associated with the spontaneous neoplastic transformation of cells in vitro.

PHS-6040
(Rev. 10-76)

Project Description:

This project consists of the following studies:

Study 1: The cytology of cells undergoing spontaneous neoplastic transformation in culture.

Objective: To study the progressive cytologic changes in 13 cell lines derived from embryos of the rat, hamster & mouse during long-term culture and to establish more quantitative, objective cytologic criteria for neoplastic transformation of cells in vitro and a systematic approach to in vitro diagnosis of neoplastic state.

Methods Employed: A combination of cytologic criteria previously described and validated for C3H mouse cell cultures is used to study individual cells at high power as well as colony morphology at low power. Colonies are generated by scraping a confluent monolayer with a small piece of perforated cellophane and the resulting suspension of small cell clumps is placed in a Leighton tube containing a glass coverslip. The clumps are allowed to adhere and spread on the coverslip for 24 hrs after which time the coverslip is fixed and stained with Giemsa. Cultures were examined for 19 cytologic abnormalities. The percentage of cells or colonies showing each of the abnormal properties was recorded.

Major Findings: Five of the 19 cytologic criteria were found to correlate with neoplastic transformation in all cell lines. These were cytoplasmic basophilia, reduced cytoplasmic spreading on substrate, cording, high nuclear: cytoplasmic ratio, and clumping.

Proposed Course: This study has been completed and a manuscript is in press. Since each of the 5 cytologic criteria is amenable to quantitation or computerized image analysis, an attempt is now underway to program a computer to make objective, quantifiable measurents of the 5 cytologic properties enumerated above.

Study 2: Measurement of cell orientation.

Objective: To develop methods for measuring the orientation of cells as they migrate from a microdrop.

Methods Used: See last year's report.

Major Findings: Cell orientation in colonies of neoplastic and nonneoplastic cells of common origin as determined by computerized image analysis were not significantly different.

Significance: A criterion of neoplastic state frequently used is the loss of parallel orientation. The present study provided a means for quantifying this parameter.

Proposed Course: This will be temporarily discontinued.

Study 3: The cytology of rat liver epithelial cells.

Objective: To identify cytologic characteristics of cultured rat liver epithelial cells that may be directly or indirectly associated with their neoplastic state.

Major Findings: Twenty-one coded cultures of epithelial cells derived from rat liver were diagnosed for neoplastic state from 24 hr. coverslip preparations. In addition, a correlation analysis was carried out to determine which of 15 cytologic criteria correlated best with neoplastic state in 17 of the lines examined. Nineteen of twenty-one coverslip preparations of the rat liver cells were correctly diagnosed. Correlation analysis indicated that 2 of the 15 cytologic characteristics correlated with tumorigenicity. They were increased cytoplasmic basophilia and increased nuclear: cytoplasmic ratio. A manuscript has been completed and submitted for publication.

Significance: Previous studies in this laboratory had been directed toward formulating diagnostic criteria for transformation of fibroblasts in culture. With increasing emphasis in our program on human epithelial cells for carcinogenesis studies, it was important to establish cytologic criteria for epithelial cells transformed either spontaneously or by chemical carcinogens.

Proposed Course: Cytologic criteria found in these earlier studies to be of diagnostic value will be quantified by means of computerized image analysis and applied to transformed human epithelial cells in culture as these become available.

Publications:

1. Sanford, K.K., Handleman, S.L. and Jones, G.M.: Morphology and serum dependence of cloned lines undergoing spontaneous neoplastic transformation in culture. Cancer Res. 37: 821-830, 1977.

2. Tucker, R.W., Sanford, K.K., Handleman, S.L. and Jones, G.M.: Colony morphology and growth in agarose as tests for spontaneous neoplastic transformation in vitro. Cancer Res. 37: 1517-1579, 1977.

3. Handleman, S.L. and Sanford, K.K.: The cytology of spontaneous neoplastic transformation in culture. In Vitro, in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5239-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Carcinomatous Change in Human Cells Studied In Vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. H. Fox	Scientist	LB NCI
Other:	K. K. Sanford	Head, Cell Physiology & Oncogenesis Section	LB NCI
	F. M. Price	Research Biologist	LB NCI
	C. U. Garrison	Laboratory Technician	LB NCI

COOPERATING UNITS (if any) J.A. Dvorak, Lab. of Parasitic Diseases, NIAID: T. Caspersson, Karolinska Institute, Stockholm, Sweden; R.E. Tarone, Biometrics Branch, NCI; Kudynowski, J.K., Karolinska Institute; Auer, G., Karolinska Institute; Kakunaga, T. Chemistry Branch, NCI

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Laboratory of Biochemistry

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Cell Physiology & Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.0	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to study changes that occur in differentiated human epithelial cells accompanying their transformation in culture using cytologic chemical and physiological parameters; to establish a system for transformation of human cells and to use such a system for estimating the likelihood of neoplastic transformation in vivo.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: To study the changes that occur in differentiated human epithelial cells accompanying transformation using morphological, chemical and physiological parameters. Further, to establish a system for transformation of human cells at will and to use such a system for estimating the likelihood of neoplastic transformation in vivo.

Methods Employed: The methods to be employed will be described under each study heading.

Study 1. Neoplastic transformation of human epithelium in vitro.

Objective: The first phase of this study was an attempt to determine the effects of polycyclic hydrocarbon carcinogens on human epithelial cells in vitro, to investigate the differences in metabolism of benzo(a)pyrene (BP) in epithelial cells from different patients, and to contrast that metabolism with BP metabolism by cultured fibroblasts from the same donor.

Major Findings: The major findings concerning this phase of the study were reported in last year's annual report and have been published.

Study 2. Morphology of transformation in human and rodent cells.

Objective: To quantify some of the cytologic changes that occur in human and rodent cells undergoing neoplastic transformation in culture.

Major Findings:

Populations from seven pairs of neoplastic and nonneoplastic rodent cell lines of common origin were compared for evidence of a change in morphological parameters accompanying neoplastic transformation. Projected cytoplasmic area, projected nuclear area, cell dry mass, nuclear dry mass, whole-cell absorption at 265 nm, and nuclear absorption of 265 nm were determined. Neoplastic transformation was consistently accompanied by three morphological features: (a) decrease in projected area of the cytoplasm (b) decrease in projected area of the nucleus and (c) decrease in dry mass of the cytoplasm. In every case the mean ratio of cytoplasm to nuclear mass was greater for the nonneoplastic than the corresponding neoplastic line while nuclear mass remained approximately the same. Thus neoplastic cells have smaller nuclear area but not necessarily a decreased nuclear mass. Ultraviolet absorption was generally less in the neoplastic than nonneoplastic cells.

Paired cultures of glutaraldehyde-fixed nonneoplastic and chemically transformed human fibroblasts were examined by quantitative microscopy. Projected cytoplasmic and nuclear areas, dry mass of cytoplasm, nuclei and whole cells, estimation of integrated cytoplasmic thickness and protein and DNA in Feulgen and naphthol-yellow stained preparations were measured. A highly significant decrease in projected area of the cytoplasm

and some decrease in projected nuclear area were observed in the transformed as compared with their nonneoplastic counterparts. The neoplastic cells also had significantly less dry mass due almost entirely to the cytoplasm since nuclear dry mass values were similar. While there was deviation in the ploidy level of the two cell types, there was little evidence that the mode was greater than diploid. Protein concentration was somewhat higher in the neoplastic cells, and the concentration of cytoplasmic protein per unit of area was considerably higher in the neoplastic cells.

Significance: These results on paired neoplastic and nonneoplastic cells of common origin provide quantitative evidence that neoplastic transformation of both rodent and human fibroblasts in culture is accompanied by morphologic change. The results indicate that one characteristic of neoplastic cell populations (i.e. the decrease in projected area and mass of lamellar cytoplasm) could be explained by decreased elaboration of cellular membrane resulting in a compact cell in which the cellular components are drawn together more closely and protein concentration is higher per unit area than in nonneoplastic fibroblasts. Such spatial disorganization of cell components might underlie the altered growth regulation in neoplastic cells.

Publications:

1. Fox, C.H., Dvorak, J.A., Sanford, K.K.: Cytometric analysis of neoplastic transformation of vertebrate cell populations. Cancer Res. 36: 1556-1561, 1976.

2. Fox, C.H., Caspersson, T., Kudynowski, J., Sanford, K.K. and Tarone, R.E.: Morphometric analysis of neoplastic transformation in rodent fibroblast cell lines. Cancer Res. 37: 892-897, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 5238-08 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Estrogen on Gene Expression in the Avian Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. G. Deeley	Visiting Scientist	LB NCI
OTHER:	R. F. Goldberger	Medical Officer (Chief)	LB NCI
	K. P. Mullinix	Research Chemist	LB NCI
	M. B. Meyers	Research Chemist	LB NCI
	W. Wetekam	Visiting Fellow	LB NCI
	J. Gordon	Research Associate	LB NCI
	K. Kent	Chemist	LB NCI
	J. D. Eldridge	Biologist	LB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Biochemistry

SECTION
Cellular Regulation Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	4.5	2.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work described herein consists of the development of a large scale purification procedure for the isolation of intact vitellogenin mRNA from the livers of estrogen-treated roosters. The purified mRNA has been translated into a full-size vitellogenin polypeptide and its size has been determined both by electrophoresis in agarose gels and by contour length measurements in the electron microscope. Vitellogenin complementary DNA of high specific activity has been prepared and used as a probe to examine the kinetics of induction of vitellogenin mRNA synthesis in rooster liver in response to treatment with estrogen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the mechanisms by which estrogen regulates the synthesis of vitellogenin, the precursor of the egg yolk phosphoproteins, lipovitellin and phosvitin, and to determine the mechanism by which vitellogenin is cleaved to yield lipovitellin and phosvitin.

Methods Employed: A procedure has been developed which allows the isolation of large quantities of intact RNA from the livers of estrogen-treated roosters. The initial extraction procedure utilizes high concentrations of guanidine·HCl in the presence of a reducing agent. The RNA preparations obtained by this procedure have been examined under rigorously denaturing conditions (by electrophoresis on agarose gels in the presence of methyl mercury), and have been found to show markedly less degradation than RNA prepared by a variety of other procedures. Vitellogenin mRNA has been purified and albumin mRNA partially purified from these preparations by a combination of affinity chromatography and centrifugation through isokinetic sucrose gradients under dissociating conditions. Recoveries of vitellogenin mRNA from commercially available affinity matrices was found to be very poor. A much more effective matrix has been synthesized by coupling long tracts of poly(U) to the surface of Sephadex beads. Using this affinity matrix and fractionation through several sucrose gradients it has been possible to purify vitellogenin mRNA with an overall yield of 20-30%. The homogeneity and size of the purified vitellogenin mRNA has been assessed by electrophoresis in agarose gels under denaturing conditions, electron microscopic examination, and hybridization analysis. Its identification as vitellogenin mRNA has been confirmed by translation of the RNA in a cell-free translation system derived from wheat germ. The cell-free synthesis system has been modified in order to obtain complete translation of exceptionally large mRNA species. The polypeptide synthesized from purified vitellogenin mRNA has been characterized by its size, its cyanogen bromide cleavage pattern and its immunological identity with authentic vitellogenin. Vitellogenin mRNA has been used as a template in studies aimed at synthesizing DNA complementary to the whole length of the vitellogenin mRNA molecule.

Major Findings: Vitellogenin mRNA has been purified to homogeneity as determined by electrophoresis in agarose gels under denaturing conditions. Its size estimated both from its electrophoretic mobility in agarose gels and contour length measurements in the electron microscope has been determined to be 2.35×10^6 daltons. The homogeneity of the RNA preparations has also been assessed by studying the hybridization of vitellogenin cDNA to its RNA template. The results obtained were consistent with those expected for a homogeneous preparation containing a single species the size of vitellogenin mRNA. It has been possible to translate purified vitellogenin mRNA into a polypeptide the size of complete vitellogenin monomer (MW 240,000). In addition, the *in vitro* product has been shown to react with purified monospecific anti-vitellogenin antibody and on cleavage with cyanogen bromide to yield an extremely unusual peptide characteristic of vitellogenin. It has been demonstrated that this peptide (MW 90,000) contains the two phosphoserine rich-polypeptides found in egg yolk, the phosvitins, which are derived *in vivo* by specific proteolysis of vitellogenin. Experiments designed to measure the kinetics of synthesis

of vitellogenin in vitro have been used to localize the phosvitins in the COOH-terminal portion of the vitellogenin polypeptide. The size estimates of vitellogenin mRNA indicate that it is approximately 10% larger than the minimum size required to specify the vitellogenin polypeptide as it is isolated from the serum.

Radioactive complementary DNA synthesized from purified vitellogenin mRNA has been used as a sensitive and direct means of measuring the concentration of vitellogenin mRNA in rooster liver, both prior to treatment with estrogen and at various times after treatment. It was found that the livers of normal roosters contained 0-5 molecules of vitellogenin mRNA per cell. Following a single injection of estradiol, the concentration of vitellogenin mRNA increased linearly for 24 hours and reached a maximum concentration (at 3 days) of approximately 10,000 molecules per cell. Thereafter, the concentration declined, decreasing to approximately 400 molecules per cell at 10 days and to less than 100 molecules per cell at 14 days. Thus, the induction of vitellogenin synthesis in the rooster appears to involve primary activation of a previously dormant gene in a tissue that is already differentiated and metabolically active. This activation process is not accompanied by the cellular proliferation found in other systems regulated by steroid hormones and which exhibit a response of this magnitude, such as the synthesis of ovalbumin in the chicken oviduct.

Significance to Cancer Research: It can be assumed that at least some forms of cancer can be attributed to aberrations in the mechanisms that control gene expression. At present, we have minimal knowledge of the processes that control the expression of genes at the transcriptional or the translational level in either the normal or cancerous cell. One possible way to study these processes is to examine biochemically the ways in which certain agents can affect specific gene expression. An ideal system for these studies is one in which hormones that can regulate the synthesis of specific proteins are employed. In the studies described above, estrogen is used to induce the synthesis of vitellogenin in the rooster, an animal in which the vitellogenin gene is completely repressed in the normal condition. In addition, the synthesis of vitellogenin is appropriate for the study of controls of regulation of post-translational modification of proteins, since vitellogenin is a protein that is phosphorylated and glycosylated after or during its translation and it is presently not known how or to what extent post-translational modifications influence specific protein synthesis and transport.

Proposed Course of Research: Vitellogenin cDNA will be used to compare the kinetics of the early phase of vitellogenin mRNA synthesis in response to both primary and secondary treatments with estrogen. These experiments will be directed towards elucidation of the mechanism involved in the enhanced vitellogenic response found on repeated treatment of the rooster with estrogen. The response of the liver to estrogen treatment will also be characterized by complexity analyses of the total RNA population of the liver at various times after treatment with estrogen. These experiments may yield evidence of the existence of several abundant species of mRNA which are induced by treatment with estradiol and may provide a cDNA probe specific for a set of genes re-

gulated by a common mechanism. The purification of albumin mRNA will be completed and the corresponding cDNA will be prepared. The availability of both albumin cDNA and vitellogenin cDNA will allow the specificity of cell-free transcription systems, currently being developed, to be tested. Permission has been obtained (MUA-16) to clone both the albumin and vitellogenin genes. Purified preparations of both RNA species will be used as templates to synthesize double-stranded DNA fragments suitable for insertion into an approved cloning vector. Work will begin on the fractionation of the chicken genome with a view to cloning large fragments of DNA containing both the structural and regulatory elements of either the vitellogenin or albumin genes. These DNA fragments will ultimately be used in a reconstituted in vitro transcription system utilizing homologous RNA polymerase, histones and nonhistone proteins. This system will be used to study the molecular mechanisms involved in regulation of expression of the vitellogenin and albumin genes.

Publications:

Mullinix, K.P., Deeley, R.G., Wetekam, W., and Goldberger, R.F.: Effect of estrogen on gene expression in the avian liver. In McMahon, D. and Fox, C.F. (Eds.): Developmental Biology. Menlo Park, W.A. Benjamin Press, 1975, pp. 409-420.

Wetekam, W., Mullinix, K.P., Deeley, R.G., Kronenberg, H.M., Eldrigde, J.D., Meyers, M., and Goldberger, R.F.: Effect of estrogen on gene expression: Purification of vitellogenin messenger RNA. Proc. Nat. Acad. Sci. USA 72: 3364-3368, 1975.

Deeley, R.G., Mullinix, K.P., Wetekam, W., Kronenberg, H.M., Eldridge, J.D., Meyers, M., and Goldberger, R.F.: Vitellogenin synthesis in the avian liver: Vitellogenin is the precursor of the egg yolk phosphoproteins. J. Biol. Chem. 250: 9060-9066, 1975.

Mullinix, K.P., Wetekam, W., Deeley, R.G., Gordon, J.I., Meyers, M., Kent, K.A., and Goldberger, R.F.: Induction of vitellogenin synthesis by estrogen in avian liver: Relationship between level of vitellogenin mRNA and vitellogenin synthesis. Proc. Nat. Acad. Sci. USA 73: 1442-1446, 1976.

Wetekam, W. Deeley, R.G., Mullinix, K.P., Gordon, J.I., Meyers, M., Kent, K.A., and Goldberger, R.F.: Effect of estrogen on gene expression: Vitellogenin synthesis may be regulated at the level of transcription and translation. In Nierlich, D.P., Rutter, W.J., and Fox, C.F. (Eds.): Molecular Mechanisms in the Control of Gene Expression. New York, Academic Press, 1976, pp. 349-354.

Goldberger, R.F., and Deeley, R.G.: Autogenous regulation of gene expression. In Marzluf, G.A. and Copeland, J.C. (Eds.): Regulatory Biology. Columbus, Ohio State University Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 5240-03 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The synthesis of vitellogenin by avian liver cells in culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. G. Deeley	Visiting Scientist	LB NCI
OTHER:	J. D. Eldridge	Biologist	LB NCI
	R. F. Goldberger	Medical Officer (Chief)	LB NCI
	C. H. Fox	Senior Scientist	LB NCI
	F. Price	Research Biologist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Cellular Regulation Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To establish a primary avian liver cell culture system in order to further elucidate the mechanism of estradiol induction of the egg yolk phosphoprotein, vitellogenin.

Methods Employed: Organ cultures have been prepared from the finely minced livers of nine day old chicks and have been maintained in culture for several weeks in a synthetic medium supplemented with 10% fetal bovine serum. In these initial experiments the use of proteolytic enzymes, to facilitate the dispersal of individual cells, has been avoided because of the possibility of destroying estrogen receptors on the cell surface.

Experiments have been carried out with cultures made from the livers of chicks which have been treated with estrogen and which were synthesizing vitellogenin when sacrificed. Similar cultures have also been prepared from the livers of untreated chicks. The cultures made from the livers of estrogen treated chicks have been maintained in a medium containing 10 M estradiol, while the cultures from untreated chicks have been maintained in a similar medium both in the presence and absence of estradiol. The polypeptides synthesized by these cultures at various times during the first two weeks in culture have been labelled with H-leucine and PO . The radioactively labelled polypeptides secreted into the medium have been examined by electrophoresis on acrylamide gels containing SDS and by various immunological techniques.

Major Findings: Chicken liver cultures prepared in the fashion outlined above continue to synthesize and secrete polypeptides for several weeks; during this period some cell division occurs. The major secreted polypeptide has the same electrophoretic mobility on SDS-acrylamide gels as albumin. Cultures prepared from the livers of chicks which have been treated with estrogen continue to synthesize intact, phosphorylated vitellogenin for the first 24 to 48 hours in cultures. After approximately three days in culture, the synthesis of intact vitellogenin polypeptides (molecular weight 240,000) is not detectable. However, two polypeptides (molecular weights 180,000 and 30,000) which may be generated by proteolytic cleavage of intact vitellogenin continue to be synthesized. These polypeptides are being characterized immunologically. The synthesis of two similar polypeptides can be induced in cultures prepared from the livers of untreated chicks by the addition of 10 M estradiol to the culture medium. So far it has not been possible to detect the synthesis of intact vitellogenin in cultures prepared from the livers of untreated chicks.

Significance to Cancer Research: The mechanism by which the expression of particular genes is regulated remains, in large part, unclear. We hope that study of the molecular mechanism of gene expression in this system will provide a better understanding of the role of steroid hormones in such regulation.

Proposed Course of Research: Terminated (July 1976).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 5242-01 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Organization of Estrogen-Responsive Genes in the Avian Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: F. C. Eden Staff Fellow LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to determine the actual number of different structural genes in the avian liver whose transcription is altered by administration of estrogen and to provide a detailed description of the organization of these estrogen-responsive genes in the avian DNA.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Administration of estrogen to roosters results in a dramatic increase in biosynthetic activity in the liver with production of the egg yolk protein, vitellogenin, as the most prominent feature of the estrogen response. The aim of this project is to determine the actual number of different structural genes in the avian liver whose transcription is altered by administration of estrogen and to provide a detailed description of the organization of these estrogen-responsive genes in the avian DNA.

Methods Employed: To determine the prominent features of sequence organization in chicken DNA, the reassociation kinetics of short fragments of chicken DNA were measured by hydroxyapatite chromatography. As does most eukaryotic genomes, chicken DNA contains repetitive and single copy DNA sequences. To study the arrangement of these sequences, short, unlabeled fragments of chicken DNA were reassociated with trace amounts of radioactive fragments ranging in size from 200 nucleotides (nt) to 18,000 nt in length. The hydroxyapatite binding of radioactive fragments of various lengths was determined after reassociation of repetitive sequence elements. An increase in hydroxyapatite binding via repetitive sequences as a function of tracer fragment length provides direct evidence for interspersion of repetitive and single copy sequence elements and indicates what fraction of the genome is interspersed as well as the average spacing between repeated sequences. Using this information, the chicken genome was preparatively fractionated into single copy and repetitive sequences. The single copy DNA fraction was labeled *in vitro* to high specific activity and used in the studies described below.

To quantitate the number of structural genes expressed in liver which has not experienced estrogen stimulation, total RNA was extracted from the livers of 6-8 week old roosters. A second group of roosters received a series of injections of estradiol and when vitellogenin synthesis reached maximal levels, the livers were excised and RNA isolated as above. Each total RNA preparation was hybridized with single copy DNA using an RNA excess of 100,000-fold. From the saturation plateau of the hybridization reactions, we can calculate directly the number of structural genes expressed in stimulated and unstimulated liver.

Major Findings: From the reassociation kinetics of short fragments of chicken DNA measured by hydroxyapatite chromatography we have determined that 10-12% of chicken DNA sequences are repeated about 200-fold. The remaining 88-90% of chicken DNA is composed of single copy sequences. From the reassociation kinetics of an isolated single copy tracer fraction with excess total DNA we calculate that the kinetic complexity of chicken single copy DNA is 2.1 pg, or about 2×10^9 ntp. Thus, the chicken genome contains sufficient single copy sequence to code for about 10^6 structural genes, each 2000 ntp. in length.

From the hydroxyapatite binding of DNA fragments of various lengths via repetitive duplexes we have determined that about 40% of the chicken genome bears an alternating arrangement of single copy and repetitive sequences with an average spacing of about 4000 ntp. An additional 10-15% of the genome appears to contain a wider spacing of repetitive sequence elements. The remaining 45-50% of the genome consists of single copy sequences uninterrupted by repetitive sequence elements for a distance of at least 18,000 ntp. Preliminary observations using partially reassociated 7000 ntp. fragments of chicken DNA examined with the electron microscope confirm these general features of sequence organization.

We are presently isolating those single copy sequences that are most closely interspersed with repetitive sequence elements. These sequences should constitute a small fraction of the total sequence complexity and will be used to determine whether a functionally related subset of single copy sequences - the estrogen-responsive genes in rooster liver - enjoy a preferential location adjacent to repetitive sequences. This information will indicate whether proximity to repeated sequences plays a role in the coordinated expression of estrogen-responsive genes.

To isolate those genes in rooster liver whose transcription is altered (turned on or off) by estrogen, we are preparing materials for a two-step preparative separation. Total RNA from stimulated liver will be hybridized to saturation with trace amounts of radioactive single copy DNA. The DNA will be re-isolated from the hybrids and will represent those genes expressed in estrogen-stimulated liver at concentrations of one molecule per cell or greater. Preliminary measurements on an analytical scale indicate that the RNA in stimulated liver represents about 6% of the total single copy sequences, or the equivalent of about 60,000 structural genes of average length 2000 ntp. This DNA fraction will then be hybridized again with an excess of unstimulated total RNA from unstimulated liver and the hydroxyapatite unbound fraction will be isolated. This fraction represents those genes whose transcription was newly initiated in response to estrogen stimulation. The limits of detection for transcriptional alteration in this experiment will be in the range of 1000-2000 structural genes. If the magnitude of the estrogen-induced change (increase or decrease) in the number of genes expressed exceeds this number, we will be able to preparatively isolate the estrogen-responsive genes. We can then determine whether these same genes are activated or repressed by estrogen in other estrogen-responsive tissues, such as chick oviduct. We can also determine whether these genes are expressed in other tissues that show no apparent response to estrogen. The results of these experiments will establish the magnitude of the estrogen response in chicken liver and indicate clearly whether hormonal stimulation alters the expression of the same genes in different tissue or whether there is clear tissue-specificity in the response to the hormone at the level of primary transcription.

Significance to Cancer Research: The basic mechanisms whereby differentiated cells undergo alterations in gene expression in response to extracellular stimuli are of direct relevance to understanding the establishment of malignant phenotype. We have chosen to study the structural features in DNA sequence

organization that are involved in the coordinated alteration of cell phenotype in a hormone-responsive tissue; this information will apply directly in the search for the underlying structural features of animal genomes that are involved in differentiation in normal cells which also could form the structural basis for transcriptional changes associated with malignancy.

Proposed Course of Research: Following preparative isolation of those genes that are involved in the estrogen response in avian liver, we will characterize these genes in a number of respects.

1) Do these genes find expression in other tissues that do or do not respond to estrogen?

2) The induction of vitellogenin synthesis in the liver by estrogen follows a predictable course of stimulation, followed by a decline in synthesis if estrogen is not re-administered. However, a second injection of estrogen results in a more rapid and more dramatic response in terms of vitellogenin synthesis, suggesting that there are some long-term effects of the hormone. We wish to determine whether a large group of genes is regulated coordinately with the vitellogenin gene in the time course of induction and decline, or whether there are genes whose expression continues after the decline in vitellogenin synthesis.

3) A prominent feature of DNA sequence organization in most eukaryotes is the interspersion of single copy and repetitive DNA sequences in a regular alternating pattern. The functional significance of this pattern is not known. The availability of a DNA fraction representing a functionally related set of genes will allow investigation of the organization of these particular genes in chicken DNA. For example, the preferential location of estrogen-reponsive genes next to repeated sequences would provide evidence for the involvement of repetitive sequences in the regulation of gene expression.

4) Although eukaryotic genomes typically contain sufficient coding capacity for more than a million structural genes, only a small fraction of this genetic information finds expression in any given tissue. This fact raises the possibility that some of the single copy sequence may not specify proteins but may serve some other unspecified function. We wish to determine whether genes whose function has been established are conserved in evolution relative to total single copy sequences. Accordingly, we have undertaken a survey of the single-copy sequence relatedness of chicken DNA with other avian DNAs to locate birds which share only a small fraction of their sequences with chicken sequences. Extensive hybridization of the estrogen-responsive genes with such distant avian species would provide an indication of conservation of a functional subset of the genome.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 5243-01 LB

PERIOD COVERED July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

In vitro synthesis of vitellogenin messenger RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. P. Mullinix	Research Chemist	LB NCI
OTHER:	J. L. Christmann	Staff Fellow	LB NCI
	J. D. Eldridge	Biologist	LB NCI
	M. B. Meyers	Research Chemist	LB NCI
	R.F. Goldberger	Medical Officer (Chief)	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 3	PROFESSIONAL: 2	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed in vitro systems to use in the study of mechanisms by which estrogen influences specific gene expression. These systems include in vitro transcription in nuclei and chromatin from livers of normal and estrogen-treated roosters and in vitro transcription of DNA by DNA-dependent RNA polymerase isolated from rooster liver.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: In a previous study we showed that the administration of estrogen to roosters causes the de novo synthesis of vitellogenin messenger RNA in the liver. This mRNA is not synthesized by normal rooster. Thus, induction of the synthesis of vitellogenin mRNA by estrogen is an ideal model system in which to study the mechanisms by which the steroid hormone, estrogen, induces the expression of an otherwise inactive gene. We have attempted to characterize several in vitro systems that we will use to study expression of the vitellogenin gene. This work includes studies on transcription in isolated nuclei, in chromatin, and by DNA-dependent RNA polymerase.

Methods Employed: We have developed a procedure for isolating nuclei that are active in transcription from rooster liver. This procedure involves the homogenization of liver in isotonic sucrose and the purification of the nuclei. We have also begun to study transcription in chromatin from normal and estrogen-stimulated roosters. Nuclei are isolated and chromatin is prepared by lysis of nuclei in hypotonic medium. The chromatin is not sheared prior to its use as a transcriptional complex and the chromatin-directed RNA synthesis that we have studied has utilized only the endogenous, chromatin-associated DNA-dependent RNA polymerase. We have begun to assess methods to monitor RNA that is synthesized in vitro. The methods that we have considered involve isolation of RNA and hybridization of the isolated RNA to complementary DNA (cDNA) made to purified vitellogenin mRNA (see Project NCI ZO1 CB 5238-08 LB). We have labeled RNA synthesized in vitro with ^{3}H-UTP and have purified RNA from nuclei. By this approach, endogenous RNA and newly synthesized RNA are present in the final product. In addition, we have labeled newly synthesized RNA with both ^{3}H-UTP and Hg-CTP. Following the synthesis, RNA is deproteinized and the RNA that was synthesized in vitro is isolated by chromatography on sulfhydryl-sepharose columns under conditions in which mercury-labeled RNA binds to the support whereas endogenous RNA does not bind. The mercury-labeled RNA is then eluted from the support, thus yielding a fraction of RNA containing only newly synthesized RNA. The latter method has the advantage of theoretically being able to separate RNA synthesized de novo from endogenous RNA but we have found technical problems in its implementation. Finally, we have begun a study to find methods to solubilize the DNA-dependent RNA polymerases from liver nuclei of normal and estrogen-treated roosters. These enzymes will be used in transcription experiments utilizing both chromatin and DNA as templates for RNA synthesis. Most methods currently employed to solubilize RNA polymerases, removing them from nuclear DNA, involve extensive sonication. These procedures result in considerable loss of enzyme activity and the RNA polymerases obtained have many properties which are probably not those found in vivo. Thus, we think that it is necessary to find different methods to isolate RNA polymerase that will yield enzymes more comparable to those found in vivo.

Major Findings: Nuclei isolated by our procedures from rooster liver are pure as measured by phase contrast microscopy and are very active in transcription. The conditions used for RNA synthesis involve the use of relatively high levels of nucleotide triphosphates, a triphosphate generating system (phosphocreatine

and phosphocreatine kinase) and rat liver cytosol ribonuclease inhibitor. The ribonuclease inhibitor is necessary to retard the breakdown of RNA due to the very high levels of ribonuclease present in preparations of rooster liver nuclei. The synthesis of RNA is linear for approximately 30 minutes. Both DNA dependent RNA polymerases I and II are active in our preparations, 62% of the synthesis being due to RNA polymerase II as measured by the sensitivity of this synthesis to α-amanitin. The nuclei incorporate approximately 4 picomoles of UMP into RNA per μg of nuclear DNA in 10 minutes of incubation at 25°C, a level of synthesis consistent with some reported in the literature for nuclei from other sources. We have found that nuclei from estrogen-treated rooster liver are approximately twice as active in transcription as are those from livers of untreated roosters. We have had considerable difficulty in isolating high molecular weight RNA from our preparations, since the levels of ribonuclease in these nuclei are so high. We are still in the process of trying to overcome this problem. Chromatin isolated from rooster liver nuclei by our procedures is also active in transcription, utilizing the endogenous chromatin-associated RNA polymerases. The chromatin directs RNA synthesis to the extent of incorporation of 5 pmoles of UMP into RNA in 10 minutes of incubation at 25°C. This synthesis is 50% inhibited by α-amanitin. We have begun to monitor RNA synthesis utilizing mercurated nucleotides. We find certain problems with this technique. The synthesis of RNA in nuclei is inhibited by approximately 50% when mercurated CTP is used and the isolated RNA is smaller than 6 S. In order to investigate further the effects of mercurated nucleotides on RNA synthesis we have investigated transcription of phage DNA by E. coli RNA polymerase by determining the levels of synthesis of RNA with and without mercurated nucleotides and by examining the size distribution of synthesized RNA. We found a 40% inhibition of RNA synthesis utilizing a mixture of 50% CTP and 50% Hg-CTP along with ^{3}H-UTP, ATP and GTP. In addition, the size distribution of Hg-labeled RNA differs from the well characterized pattern of RNA molecules synthesized with non-mercurated nucleotides. At present we do not plan to utilize mercurated nucleotides to examine specific RNA synthesis in vitro but will use radioactively labeled nucleotides and adjust hybridization conditions to accommodate the endogenous RNA present in nuclei and chromatin. As an approach toward reconstructing a transcription system utilizing the vitellogenin gene DNA, RNA polymerase, and other necessary proteins, we have solubilized RNA polymerase from lysed nuclei by digesting DNA with DNAse bound to sepharose, thus liberating the DNA polymerase from its template. Preliminary data suggest that this is an efficient method of solubilizing both RNA polymerases I and II. These enzymes are active in transcription and utilize native DNA as the preferred template.

Significance to Cancer Research: The elucidation of mechanisms that control biosynthetic processes is crucial to understanding cancer. Study of the means by which estrogen influences RNA synthesis will help determine how RNA synthesis is controlled. The study of vitellogenin mRNA synthesis in rooster liver has the great advantage that we can study the expression of a normally dormant gene in a differentiated tissue that has the capability of synthesizing RNA of a very large number of types.

Proposed Course of Research: We will study specific transcription in the *in vitro* systems we have developed by means of hybridization to vitellogenin DNA.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5231-03 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Subunit Interactions in Enzyme Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Klee	Research Chemist	LB NCI
Other:	J. Craine	Senior Staff Fellow	LB NCI
	E. Barbehenn	Staff Fellow	LB NCI
	P. Richman	Staff Fellow	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.4	3.0	1.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The quaternary structure of M. luteus polynucleotide phosphorylase has been determined. The enzyme is an oligomer composed of three identical subunits. The enzyme undergoes conformational changes upon binding of oligo or polynucleotides which are responsible for the electrophoretic heterogeneity.

The interaction of cAMP phosphodiesterase with its Ca^{2+}-dependent activator protein has been utilized to purify the activator-dependent form of the enzyme and an activator binding protein which inhibits the activation of the enzyme.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the functional role of subunits and protein-protein interactions in two enzymatic systems: polynucleotide phosphorylase and cAMP phosphodiesterase.

Methods Employed: The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.
Polynucleotide Phosphorylase.

Structure function relationship. The multiple molecular forms of primer-independent polynucleotide phosphorylase have been characterized. The predominant species, with a molecular weight of 250,000 appear to be a oligomer composed of 3 identical subunits by SDS and acid urea gel analysis before and after cross linking of the polypeptides with dimethyl suberimidate. This oligomeric protein exists in multiple conformational states induced by oligo and polynucleotide binding. These multiple states are responsible for the electrophoretic microheterogeneity observed. The enzyme is also found as a high molecular complex (400,000 M.W.) with another polypeptide chain which is readily dissociable under electrophoretic conditions.

Mechanism of action. During de novo polymerization of ADP or UDP the enzyme was shown to synthetize long chains of defined chain length prior to release the formed product. The exact size of the polymer and the mechanism by which the enzyme can determine the proper number of nucleotide residues to be incorparated is presently under study (E. Barbehenn and C. Klee).

Regulation of cAMP Phosphodiesterase.

Activator dependent cyclic AMP phosphodiesterase has been purified 3000 fold to a specific activity of 140 units/mg by affinity chromatography on activator protein coupled to sepharose, gel filtration and hydroxylapatite chromatography. A number of proteins which are devoid of enzyme activity are also adsorbed specifically on the activator-Sepharose. One of these proteins composed of two polypeptides chains of 65,000 and 15,000 MW acts as a specific inhibitor of the activation of the enzyme by the activator protein. Thus activation of cAMP phosphodiesterase by the Ca^{2+} -dependent activator may be controlled by interactions with a third component of the enzyme complex. In view of the similarity of activator protein with the Ca^{2+} binding component of the troponin complex troponin C, the relationship of these proteins to the contractile proteins of the cells is also being investigated (C. Klee and M. Krinks).

The conformation-dependent nitration of the activator protein with tetra nitromethane has confirmed our previous observation that one of the two tyrosine residues is greatly affected by Ca^{2+} binding and may be

surrounded by a cluster of carboxyl residues which are responsible for its abnormal behavior. It was also shown that nitration did not significantly modify the ability of the activator to activate cAMP phosphodiesterase (P. Richman and C. Klee).

Significance to biomedical research and the program of the Institute:

Both proteins being studied are examples of regulation of catalytic activity by protein-protein interactions. Polynucleotide phosphorylase, a well-known bacterial enzyme, also has been described in mammalian cells where its phosphorolytic activity alone has been observed. It is widely utilized for the synthesis of model nucleic acids used in nucleic acid enzymology and interferon induction, and also in the degradation of poly A sequences of mammalian mRNA. Its physiological role is still undetermined, but it would seem likely that the enzyme has an important function in nucleic acid metabolism.

cAMP phosphodiesterase, as one of the enzymes responsible for the control of cAMP levels which are critical for the regulation of cell growth and differentiation, is of considerable interest. The potential role of activator protein in mediating the cellular effects of Ca^{2+} and the functional state of contractile proteins is of obvious importance. This ubiquitous regulatory, protein could provide a link between cyclic nucleotide levels and multiple cell functions. The ability of the activator protein to regulate a large number of biological functions represents a novel mechanism with potential physiological significance.

Proposed course of research

Studies on polynucleotide phosphorylase will be directed towards the elucidation of the mechanism of action of the enzyme: particularly its ability to synthetize polymers of precise chain length in the absence of a template. The role of tightly bound adenylate kinase and ATPase activities in the initiation of polymerization will be also investigated further.

cAMP phosphodiesterase activity is regulated by two naturally occuring proteins: Ca^{2+} dependent activator and an activator binding protein as described above. The effects of other types of positive or negative effectors such as imidazole and theophylline on the regulation of the enzyme by its physiological effectors will be studied. All studies will be conducted on enzyme or regulatory proteins purified to homogeneity in order to simplify the interpretation of the results. Subunits and protein-protein interactions will be studied in order to elucidate their potential role in the complex cooperative kinetics exhibited by the enzyme and the concerted regulation mechanism of the activator protein. Subcellular localization of the various components will be studied with the aid of antibodies which also may be useful in studies of the regulation of synthesis and degradation of these protein.

Publications

1. Klee, C.B.: Conformational transition accompanying the binding of Ca^{2+} to the protein activator of 3',5'-cyclic adenosine monophosphate phosphodiesterase. Biochemistry 16: 1017-1024, 1977.

2. Craine, J.E., and Klee, C.B.: A deoxyadenylate kinase activity associated with polynucleotide phosphorylase from micrococcus luteus., Nucleic Acid. Res. 3: 2923-2938, 1976.

3. Klee, C.B., LaJohn, L.E., Kirk, K.L., and Cohen, L.A.: 2-fluorourocanic acid, a potent reversible inhibitor of urocanase. Biochem. Biophys. Res. Comm. 75: 674-681, 1977.

4. Klee, C.B.: Interactions of Ca^{2+}-dependent activator protein and troponin C with cAMP phosphodiesterase and its inhibitor. Third US-USSR symposium on myocardial metabolism, to be published by NIH, Williamsburg, Virginia, May 9-11, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CBD 5232-03 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Replication of Simian Virus 40 (SV40) DNA in Monkey Kidney Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Kay	Visiting Fellow	LB NCI
Other:	M.F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The synthesis of histones during lytic SV40 infection of BSC-1 (African Green Monkey Kidney) cells has been investigated. The synthesis of all five classes of histones was stimulated to the same extent. Post-infection an increased rate of histone synthesis is detectable several hours before SV40 DNA synthesis is measurable and the rate of histone synthesis declines at a time when SV40 DNA synthesis is still at the maximum rate. Thus histone synthesis is not strictly coupled to SV40 DNA synthesis.

The DNAs of two separately isolated, plaque-purified wild type strains of SV40 have been compared by restriction endonuclease mapping. One, 777, is significantly altered in the gene for the capsid protein, VP1. The difference was confirmed by analysis of tryptic peptides from the VP1s of both strains. Other differences in the genomes of the two strains were also detected.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. To study the synthesis of histones that accompanies SV40 infection and SV40 DNA synthesis in permissively infected monkey cells.

2. To investigate the DNA structures of two physiologically distinct SV40 "wild type" strains.

Methods Employed:

Propagation of SV40 and SV40 DNA in monkey kidney cells in tissue culture. Extraction procedures for histones using both acid and neutral (detergent) techniques. Polyacrylamide gel electrophoresis of proteins and nucleic acids. Separation of subfractions of H1 histone on columns of IRC-50 with guanidinium chloride gradients. Restriction endonuclease digestion. Electron microscope heteroduplex analysis. Tryptic digestion and separation of peptides on Aminex A-5 columns. Radioisotopic labeling of DNA and proteins. Preparative ultracentrifugation.

Major Findings:

1. We have shown that lytic SV40 infection of BSC-1 cells stimulates histone synthesis. In mock infected cells, what histone synthesis there is, and it is low, closely follows the incorporation of labeled precursor into DNA. In infected cells, however, histone synthesis in response to SV40 infection appears to begin and decline several hours before similar events can be detected in the rate of SV40 DNA synthesis. These experiments gave some indications that the synthesis of H1 histone during SV40 infection was different from that in uninfected cells. Therefore we undertook to separate different subfractions of H1 histone in order to study the possibility that one or more of them were preferentially stimulated during infection. A column of IRC-50, a weakly acidic ion exchange resin was used, eluting with a shallow gradient of guanidinium chloride. H1 was first purified by specific extraction from nuclei. Uninfected cells were labeled with ^{14}C lysine. Infected cells were labeled prior to infection with ^{14}C lysine and then with ^{3}H-lysine from 18-45 hours after infection. A stimulated subfraction would therefore have a higher $^{3}H/^{14}C$ ratio than other subfractions. Our preliminary results show that there is an H1 subfraction with a $^{3}H/^{14}C$ ratio, about twice that of other subfractions.

2. Differences between the DNAs of SV40 "wild type" variants 776 and 777 have been detected at five distinct points on the SV40 genome by restriction endonuclease analysis. The most obvious difference is a deletion of about 0.5% of the SV40 genome in 777 in the endo R·HindII/HindIII-F fragment. This has been localized more precisely within a smaller restriction fragment produced by endonuclease R·AluI (L). The deletion can then be placed between map positions 0.02 and 0.05, within the region known to code for

the major capsid protein, VP1. The tryptic peptides produced from the VP1s of 776 and 777 differ to some extent, but the differences are not easily explained by a simple deletion. Furthermore, the digestion of 777 with endonuclease AluI suggests that other peculiarities may exist within the gene for the 777 capsid protein. Other differences between the two strains were detected at three positions on the genome, 0.175-0.21, 0.54-0.585, and 0.66-0.71, within regions known to be involved in termination of transcription, coding for early functions, and the origin of replication, respectively. Earlier work in other laboratories indicated that deletions in these regions lead to viable virus. Finally, a difference between the two strains was also noted at approximately 0.8 on the genome.

Earlier work had shown that defective virus obtained from high multiplicity passaging of 777 interfered with the replication of wild type 777. Thus, these defective virions were typical of the defective, interfering virus produced by many animal virus systems. We have now demonstrated that the interference is strain specific: the defectives are much less efficient at interfering with the replication of strain 776 than with the parent, 777.

Significance to Cancer Research:

SV40 is oncogenic to some small mammals. A detailed knowledge of the events following permissive infection of monkey cells by SV40 will lead to a clearer idea of the interaction been the virus and infected cell.

The differences between the genomes of strains 777 and 776 virus are of special interest because various wild type SV40 strains are known to differ in oncogenicity.

Proposed Course of Research:

1. Terminate this project and incorporate into Z01 CBD 5237-03 LB as the aims and experimental approaches are now approaching each other.

2. The work on comparison of 777 and 776 will be terminated.

Publication:

1. LeBlanc, D.J., and Singer, M.F.: Simian virus 40 DNA replication in Nuclear monolayers. J. Virol. 20: 78-85, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5234-03 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Replication and Properties Defective - Substituted Simian Virus 40 in Permissive Monkey Kidney Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI
Other:	H. Rosenberg	Visiting Fellow	LB NCI
	D. Singer	Staff Fellow	LB NCI
	M. Nasoff	Biological Lab Technician	LB NCI

COOPERATING UNITS (if any)

1. Martin Rosenberg, Ph.D. LMB NCI
2. S. Segal, Ph.D. Section on Biochemistry of Gene Expression, LB NCI
3. E. Kuff, Ph.D. Section on Biochemistry of Gene Expression, LB NCI

LAB/BRANCH

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Nucleic Acid Enzymology Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	2.7	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A defective variant of simian virus 40 containing covalently linked DNA sequences derived from the host (African Green monkey) genome is being used to study the origin of such substituted defectives as well as the structure of monkey DNA. The nucleotide sequence of a segment of the defective genome containing monkey sequences (185 base pairs) has been determined. The segment from the defective hybridizes, in situ to both centromeric and non-centromeric regions of African Green monkey chromosomes. The sequence in the defective is derived from highly repeated monkey DNA and occurs almost 2 million times in the monkey genome. Cleavage of total monkey DNA with restriction endonuclease HindIII permits isolation of a 172 base pair long fragment representing about 7 percent of the total DNA. The nucleotide sequence of this fragment was also determined. The two fragments, one from the defective and one from monkey DNA have 156 base pairs in common. The fragment from the defective joins, on both ends, sequences not found in the segment isolated from the monkey DNA.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The discovery of variants of simian virus 40 that are substituted with DNA segments derived from the host cells, African Green monkey kidney cells, offers the opportunity to study two aspects of mammalian DNA chemistry: recombination and organization of the genome. It is the purpose of this work to exploit these variant viral genomes to study the mechanism of recombinational processes and also the structure of the monkey genome.

Methods Employed: Standard tissue culture procedures are used for the growth of monkey kidney cells and SV40. Procedures used for studying viral DNA synthesis included radioisotope tracer techniques, preparative and analytical ultracentrifugation, DNA-DNA hybridization, selective precipitation of high molecular weight DNA. Electron microscope is used to study the size and configuration of DNA and is also used to study DNA structure by heteroduplex analysis. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the preparation of pure DNA fragments of defined size. A variety of methods are used for the determination of the sequence of nucleotides in segments of DNA. These include 1) preparing a cRNA copy of the DNA segment with *E. coli* RNA polymerase, analysis of the RNA sequence by complete and partial digestion with pancreatic and T1 ribonucleases and determination of the structure of the oligoribonucleotides produced and 2) direct DNA sequencing techniques using both partial snake venom phosphodiesterase digestion of terminally labeled fragments and the chemical procedures (methylation and hydrazinolysis introduced by Maxam and Gilbert, also carried out on terminally labeled DNA fragments.

Major Findings: Two fragments containing monkey DNA sequences were isolated from a defective SV40 genome that carries host sequences in place of portions of the SV40 genome. The restriction endonuclease map of this defective was described in last year's report. The fragments each have one end produced by digestion with restriction endonuclease HindII and one produced by restriction endonuclease HindIII. The HindIII site is the junction between the two fragments within the defective. Hybridization analysis indicated that one of the fragments, called here E, contains nucleotide sequences homologous to those of highly repetitive monkey DNA. The other fragment C contains a small segment of DNA from the highly repetitive portion of monkey DNA and the remainder appears to be derived from less highly repetitive monkey sequences.

Viral DNA from a variety of independently derived SV40 defectives known to be substituted with monkey sequences was prepared and tested, by hybridization, for the presence of sequences homologous to those in fragments E and C of the original defective variant. The other defectives appear to fall into two classes: one class contains sequences homologous both to C and E, the second class contains different, non-homologous monkey sequences. Thus, these results suggest that the monkey sequences present in substituted defectives are not a random selection of monkey sequences but

may reflect some specificity in the interaction of the virus and its host.

In last year's report we mentioned that the location of the sequence in fragment E, within monkey chromosomes, was being studied by in situ hybridization. Those studies have been completed and can be summarized as follows. Radioactive cRNA prepared with isolated fragment E as template was hybridized in situ to chromosomes of primary African Green monkey kidney cells. All or part of the sequence in fragment E occurs at both centromeric and noncentromeric positions in many, but not all autosomal chromosomes. The sequence was never detected in chromosomes tentatively identified as X and Y. Of the typical 60 chromosomes, between nine and eleven hybridized with the cRNA in noncentromeric regions.

The complete nucleotide sequence of fragment E was determined using both RNA and direct DNA sequencing techniques. In addition, the 33 residues of fragment C that are adjacent to fragment E in the defective were also determined. Fragment E is 151 base pairs long. The sequence contains no discernible multiple internal repeating units as has been reported for the highly repetitive DNA of other species. The sequence does however contain a striking alternating array of blocks of purines and blocks of pyrimidines. The A-T content is 59.6 percent. The sequence has several short regions of two fold symmetry. One of these is such that it could give rise to a stem and loop structure. The sequence of fragment E also has an interesting array of sequences near it HindII end: overlapping with the HindII specific sequence is a restriction endonuclease EcoRII specific sequence, and overlapping that a sequence specific for restriction endonuclease Hph. There is no reason to expect such as array of bacterial restriction enzyme sites within this mammalian DNA. We have speculated that these symmetrical sequences, used in bacteria for restriction and modification of DNA, may be involved in recombinational events within this mammalian system.

Digestion of total monkey DNA from uninfected cells with restriction endonuclease HindIII yields a series of well defined DNA fragments of various molecular weights. The smallest, and predominant fragment (called M-1) is 172 base pairs long, and the others are whole number multiples of 172 base pairs. We have found that all those discrete fragments contain sequences that hybridize with fragment E. Therefore one of them may be the source of the monkey sequences found in fragment E. Hybridization kinetics indicates that the sequences of fragment E occur about 1.6 million times in the monkey genome, corresponding to about 5% of the total DNA. Direct quantitative determination indicated that M-1 represents about 7% of the monkey genome, and M-2, the next longer fragment, about 1.5%.

The complete nucleotide sequence of fragment M-1 was determined by the methods described for E, above. Residues 1-124 from one of its HindIII ends is identical to the first 124 base pairs starting at the HindIII end of Fragment E, thus confirming the results of the hybridizations, and indicating very extensive homology between the two. Similarly, the 33 determined residues of fragment C are identical, in 32 positions, to residues 139-172 of M-1.

Other experiments both in our laboratory and in others have indicated that the fragment we call M-1 is arranged in tandem repeats within the monkey genome. The comparison of the sequence of M-1 with the determined monkey sequences from the defective are consistent with this. Thus, in the recombinational events which gave rise to the defective, M-1 sequences were picked up starting with residue 139 (equivalent to residue 33 of C) and going through residue 172 (equivalent to residue 1 of C) continuing to residue 124 of M-1. Thus, 157 residues from M-1 appear in the defective genome. Residues 125 through 139 of M-1 are not found in the defective, nor are residues 125 through 152 of fragment E found in M-1. Thus, residue 33 of fragment C and residue 124 of fragment E represent junctions between M-1 sequences and some other sequences...either monkey, SV40, or neither.

One of the junctions, residue 124, is the center of a palindrome in the M-1 sequence...the palindrome runs from residue 117 through residue 131. Thus, the junction within fragment E occurs at the center of this palindrome.

Proposed Course of Research:

1. Sequences in the defective variant other than those in fragments C and E will be investigated in order to define the junction between monkey sequences and wild type SV40 sequences.

2. The nature of the monkey sequences, in the defective, that are not homologous to highly reiterated monkey DNA will be investigated in an effort to determine their origin within the monkey genome.

3. The nature and origin of the sequences on either side of the junctions of the M-1 sequences within fragment E will be investigated.

4. An independently derived defective variant of SV40, known to contain fragment E and C sequences will be investigated to determine the extent of homology in those sequences between the two variants, and to compare the chemical structure of the various junction points.

5. The possibility that minicircles composed of the M-1 sequence replicate in monkey cells will be investigated.

6. Attempts will be made to study whether there are proteins within monkey cells that recognize the M-1 nucleotide sequence.

Significance to Cancer Research: Interaction between oncogenic viruses and infected cells appears to be involved in certain instances of oncogenic transformation. The ability to study the interaction of the genomes of the virus and the host cell, an opportunity presented by the natural occurrence of SV40 containing monkey sequences, should help elucidate the nature of the interactions. In addition, these defective variants permit the study

of portions of the monkey genome and offer the possibility of learning something about the mechanisms of recombination in eukaryote cells.

Publications:

1. Segal, S., Garner, M., Singer, M.F. and Rosenberg, M.: In Situ hybridization of repetitive monkey genome sequences isolated from defective simian virus 40 DNA. Cell 9: 247-257, 1976.

2. Berg, P. and Singer, M.F.: Seeking wisdom in recombinant DNA research, Fed. Proc. 35: 2542-2543, 1976.

3. Singer, M.F.: Summary of the National Institutes of Health (USA) Guidelines for Recombinant DNA Research. Gene 1: 123-139, 1977.

4. Rao, G.R.K. and Singer, M.F.: Studies on a defective variant of simian virus 40 that is substituted with DNA sequences derived from monkey DNA I. Origin, properties, and purification. J. Biol. Chem. In press, 1977.

5. Rao, G.R.K. and Singer, M.F.: Studies on a defective variant of simian virus 40 that is substituted with DNA sequences derived from monkey DNA II. Structure of the DNA. J. Biol. Chem. In press, 1977.

6. Rosenberg, M., Segal, S., Kuff, E.L. and Singer, M.F.: The nucleotide sequence of repetitive monkey DNA found in defective simian virus 40. Cell In press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5237-03 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Interaction of Histones with Superhelical DNA.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. S. Singer	Staff Fellow	LB NCI
Other:	M. Bina-Stein	Staff Fellow	LB NCI
	M. F. Singer	Head, Nucleic Acid Enzymology Section	LB NCI

COOPERATING UNITS (if any)

Dr. Robert Simpson, LBC NIAMDD
Dr. T. A. Langan, University of Colorado Medical Center, Denver.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2.1	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Complexes of H1 histone and closed circular SV40 DNA were analyzed on sedimentation velocity gradients. At 0.1 M NaCl the binding of H1 to relaxed DNA is cooperative, yielding only rapidly sedimenting complexes containing an average of 40-45 molecules of H1 per molecule of relaxed DNA. Under the same conditions superhelical DNA forms two distinct complexes, a slowly sedimenting one containing 10-12 molecules of H1 per molecule of DNA and a rapidly-sedimenting, cooperatively formed complex containing between 45 and 80 molecules of H1 per molecule of DNA. Phosphorylated H1 histone forms similar complexes.

Complexes of superhelical DNA with histones H3 and H4 were also studied, using DNA relaxing enzyme, hydrodynamic measurements, and electron microscopy to study the effect of H3 and H4 on DNA conformation. It was found that H3 and H4 alone compact duplex DNA and introduce torsional constraints equivalent to that obtained with the full complement of histones H3, H4, H2a, and H2b, in intact nucleosomes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the interaction between mammalian histones and superhelical DNA in vitro and relate the interaction to the functional role of histones in DNA structure and metabolism. Emphasis is placed on SV40 DNA as a model for superhelical DNA.

Methods Employed:

Standard tissue culture techniques for growth of monkey kidney and mouse or human cells and for preparation of simian virus 40 (SV40) DNA. Subcellular fractionation, protein purification, radioisotope tracer techniques, gel electrophoresis, ultracentrifugation, nitrocellulose filter binding techniques, protein chemistry and amino acid analysis; enzymic techniques (involving polynucleotide ligase or "unwinding enzyme"), coupled with agarose gel electrophoresis for isolation and analysis of closed-circular duplex DNAs of known superhelicity; electron microscopic visualization of superhelical DNA and bound histones. In addition to radioautography, fluorography has been used to visualize radioactive material on polyacrylamide gels. Sedimentation velocity gradients used metrizamide to form gradients.

Major Findings:

Earlier we found that the extent of interaction of H1 and H5 histones with superhelical DNA increases with increasing superhelical density, regardless of whether the superhelical density is of the positive or negative sense. Furthermore, using DNA-relaxing enzyme it was shown that the interaction of duplex DNA with H1 does not induce torsional constraints into the duplex DNA structure. Studies of various polypeptide fragments of the H1 histone molecule indicated that the globular region of calf thymus H1 is involved in the discrimination between superhelical and relaxed DNA. These studies have now been extended to investigate the nature of the complexes formed between both superhelical and relaxed DNA and H1, and the stoichiometry of complex formation. These results offer an explanation for the apparent preference of H1 for superhelical DNA that was previously observed in nitrocellulose binding studies.

Complexes of H1 and circular SV40 DNA were studied by velocity gradient sedimentation analysis on metrizamide gradients. In each instance, essentially all of the H1 histone was found bound to DNA; none was free. With relaxed circular DNA, a rapidly sedimenting complex containing (at 0.1 M NaCl) between 40 and 45 moles of H1 per mole of DNA is the only complex detected. No complexes of intermediate sedimentation coefficient were observed. Thus, H1 reacts cooperatively with relaxed DNA. Superhelical DNA also interacts cooperatively with H1 histone to form rapidly sedimenting complexes; in this instance the complexes contain between 45 and 80 moles of H1 per mole of DNA. However, superhelical DNA can also form a slowly sedimenting complex with H1. This complex contains between 10 and 12 H1 molecules per molecule of DNA. The slowly sedimenting complexes

are observed at relatively low molar input ratios of H1 to DNA: at higher ratios the rapidly sedimenting complex predominates. No such complexes are observed with relaxed DNA in 0.1 M NaCl. This means that at a given molar input ratio of H1 to DNA, more superhelical DNA is complexed. Thus the original observations with the nitrocellulose filter binding assay can be explained by the ability of superhelical but not relaxed DNA to form the slowly sedimenting complex. Cooperative interaction of DNA with H1 appears to be inhibited by low salt conditions. Thus, with superhelical DNA no rapidly sedimenting complex was observed until greater than 40 mM NaCl and with relaxed DNA, both slowly sedimenting complexes and complexes sedimenting between the slowly sedimenting complex and rapidly sedimenting complexes were observed below 20 mM NaCl.

Complexes of phosphorylated H1 histone and closed circular relaxed and supercoiled DNA have also been analyzed by velocity sedimentation gradients. Qualitatively, the results were similar to those obtained with nonphosphorylated H1 histone. Thus, at all input molar ratios of phosphorylated H1 to relaxed DNA, interaction is cooperative and gives rise only to rapidly sedimenting complex, containing about 75 H1 molecules per molecule of DNA. At low input ratios of histone to superhelical DNA, interaction of phosphorylated histone and DNA yields predominantly slowly sedimenting complex with a histone to DNA ratio of 10. At higher input ratios, rapidly sedimenting complex is formed, with a molar ratio of 48-52. Four different species of phosphorylated histones were studied: phosphorylated either at residue 37 or 106, at both 37 and 106, and at the so-called "growth associated" phosphorylation sites. All four bind DNA and retain the ability to discriminate between superhelical and relaxed DNA with the same efficiency as the non-phosphorylated H1 histone. The dependence of the cooperative formation of rapidly sedimenting complexes between relaxed DNA and phosphorylated H1 histone on salt concentration is similar to that observed with non-phosphorylated H1. Furthermore, phosphorylation of H1 histone at serine 106, and to a lesser extent at serine 37, markedly increases the ability of H1 to bind superhelical DNA in the absence of added NaCl.

The eukaryotic chromatin is organized into nucleoprotein subunits (nu bodies or nucleosomes). The composition of chromatin core particles, obtained by digestion of nuclei or chromatin with micrococcal nuclease, is constant and consists of a 140 base pair (BP) length of DNA associated with two moles each of histones H2A, H2B, H3 and H4. Histone-histone and histone-DNA interactions result in folding the DNA thus decreasing the length by 5-6 times. The resulting DNA is under torsional constraints (equivalent to unwinding the DNA duplex by approximately one Watson-Crick turn per nucleosome. Certain features of the nuclease digestions and X-ray diffraction patterns of chromatin are also observed with the reconstituted complexes of arginine rich histones (H3 and H4) and DNA. We have examined other properties of the complexes of the H3+H4 histones with DNA, namely the extent of compaction, torsional constraints, and morphology.

In our study we have combined biochemical methods (sodium dodecyl sulfate and agarose gel electrophoresis, unwinding assay, etc.) along with nydrodynamic measurements (on Beckman Model E analytical ultracentrifuge)

and high resolution electron microscopy (Phillips 300). We observe that the association of 140 base pairs of DNA with a tetramer of arginine rich histones (H3 and H4) results in a partial core particle (R body). The sedimentation coefficient of the R body ($S_{20,w}$ = 9.8) is higher than that of free DNA ($S_{20,w}$ = 5.2) and that of a complex of H2A and H2B with 140 base pairs DNA ($S_{20,w}$ = 7.0). This indicated that the frictional coefficient of the R body is reduced as compared to that of protein free DNA; therefore the DNA in the complex is compacted. Morphologically, the R body resembles native core particles. Both particles are about 75A° in diameter and the central staining observed for the core particle is also observed for the R body.

Nucleoprotein complexes obtained from the association of arginine rich histones (H3 and H4) with SV40 DNA are morphologically similar to SV40 minichromosomes, which contain all four smaller histones. The complexes with superhelical DNA (DNA I) appear relaxed in electron micrographs and contain 16 ± 3 spherical particles. The DNA in each particle is calculated to be about 165 base pairs. Arginine rich histones also introduce torsional constraint into closed circular DNA, similar to that obtained with the full complement of histones (H3, H4, H2A, H2B). The alteration induced in the DNA duplex by H3+H4 histones is measured by associating H3+H4 histones with DNA I and DNA I^r and treating the complexes with relaxing enzyme. The final topological state of DNA, after deproteinization, is determined by agarose gel electrophoresis. We have observed that the resulting DNA is superhelical, regardless of whether the starting DNA is DNA I or DNA I^r. We have therefore concluded that the arginine rich histones are able to change the conformation of DNA. None of these features are observed for complexes of H2A+H2B with SV40 DNA.

We have therefore concluded that the arginine rich histones form the structural framework for folding of DNA in nucleosomes. The role of lysine rich histones in the architecture of the core particles is not clear.

Significance to Cancer Research: The role of histones in 1) the structure of chromatin and 2) the regulation of gene expression and DNA replication in eukaryotic cells is under intensive investigation in many laboratories. The relevance of the control of eukaryote gene expression to the nature of oncogenic processes has been widely discussed.

Proposed Course of Research:

1. To investigate the forms of H1 histone produced concomitant with SV40 DNA synthesis in lytically infected cells. Emphasis will be placed on distinguishing H1 histone forms that differ both in primary structure and in phosphorylation. At present the physiological significance of the various known forms of histone H1 is not understood. By using SV40 DNA replication as a model system, it is anticipated that the role of the multiple H1 histones may be elucidated.

Publications:

1. Singer, D.S., and Singer, M.F.: Studies on the interaction of H1 nistone with superhelical DNA: Characterization of the recognition and binding regions of H1 histone. Nucleic Acids Res. 3: 2531-2547, 1976.

2. Bina-Stein, M., Vogel, T., Singer, D.S., and Singer, M.F.: H5 histone and DNA-relaxing enzyme of Chicken Erythrocytes. J. Biol. Chem. 251: 7363-7366, 1976.

3. Bina-Stein, M., and Singer, M.F.: The effect of H1 histone on the action of DNA-relaxing enzyme. Nucleic Acids Res. 4: 117-127, 1977.

4. Bina-Stein, M., and Simpson, R.T.: Specific folding of DNA by H3 and H4 histones. Cell, in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 333-14 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Biochemical Basis for Defective Maturation in Myelogenous Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P I:	W. H. Evans	Research Chemist	LB NCI
OTHERS:	E. A. Peterson	Research Chemist Head, Protein Chemistry Section	LB NCI
	M. G. Mage	Immunochemist	LB NCI
	S. Wilson	Biologist	LB NCI
	F. Boctor	Visiting Fellow	LB NCI

COOPERATING UNITS (if any)

Gilbert H. Smith, Research Biologist, C/LMBGY

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at identifying the main molecular constituents and metabolic processes that distinguish normal mature granulocytes from their immature precursors and establishing which of the many steps involved in granulocyte maturation are regulated by factors present in body fluids and/or by cell-mediated factors. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested maturation steps. Biochemical analyses of molecular constituents are carried out on mature and immature granulocytes isolated from blood and bone marrow by velocity sedimentation and density centrifugation procedures developed in this

PHS-6040
(Rev. 10-76)

(Summary cont'd)
laboratory. The effects of external cell regulators on granulocyte production and differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a chemically defined culture system previously developed in this laboratory.

Major Findings:

A. Development of a Transplantable Granulocytic Leukemia in the Guinea Pig.

We are using the guinea pig hematopoietic system as a model for studies on the regulation of granulocyte production in the bone marrow. A search for possible defects in these regulatory mechanisms in leukemia could not previously be studied in the guinea pig since granulocytic leukemia in this species had not yet been reported. We have now observed the occurrence of a granulocytic leukemia in one of forty strain 13/N female guinea pigs given N-nitroso-N-butylurea continuously in their drinking water for 21 weeks. This leukemia has been successfully transplanted in this guinea pig strain for 13 transplant generations by intraperitoneal inoculation of leukemic blood or marrow cells. Macroscopically and microscopically, this leukemia resembles the chronic myelogenous form in humans. Histochemical studies showed, however, that unlike the human leukemic cells, those in the leukemic guinea pigs are alkaline phosphatase positive. Electron microscopic studies of the guinea pig leukemic cells revealed the presence of numerous intracisternal A-type particles which are not found in corresponding normal leukocytes. The development of a transplantable granulocytic leukemia in the guinea pig makes available another important model of the human disease for well-controlled biological and biochemical studies on the mechanism(s) of myeloid leukemogenesis.

B. Development of Biochemical Assays for Regulators of Granulocytic Proliferation and Differentiation in Bone Marrow.

Work continues on the isolation and purification of specific proteins from the cytoplasmic granules of granulocytic leukocytes. These proteins will be used as markers in assays for regulators of granulocyte production in normal, inflammatory and leukemic bone marrow. Using the Triton extraction and gel electrophoresis procedures reported previously we have been able to isolate and prepare antisera to granule proteins. Immuno-fluorescent methods indicate that one of these antisera shows a high degree of specificity for neutrophilic granulocytes in the presence of the heterogeneous cell types found in the bone marrow. With these specific marker proteins and related antisera it should now be possible to specifically monitor the rate of granulocyte production under various in vitro and in vivo conditions.

C. Isolation and Biochemical Characterization of Nuclei from Immature and Mature Guinea Pig Granulocytes.

As part of a study to identify the major changes in protein composition which occur in bone marrow granulocytes during cell differentiation we have

analyzed various subcellular fractions of mature and immature granulocytes for their protein components using gel electrophoretic methods. We have recently completed studies on the nuclei of such cells. Intact nuclei were isolated in high yield from enriched fractions of immature and mature guinea pig granulocytic leukocytes. These nuclei were used to determine whether any changes in synthesis and content of nuclear proteins accompany the striking increase in chromatin condensation and the nuclear lobation which occur during granulocyte maturation. The results indicate that the synthesis of nuclear proteins and the nuclear RNA content decrease markedly during granulocyte maturation. The incorporation of L-[U-^{14}C] leucine into histones of immature cells is about 25 times that of mature cells and the incorporation into non-histone proteins from immature cells is about 6 times that of mature cells. It appears that there is very little quantitative change with respect to the protein components of nuclei from immature and mature granulocytic leukocytes. No significant differences in the amounts of histone, non-histone protein or phosphoprotein between nuclei of immature and mature granulocytes could be detected. No major differences in gel electrophoretic patterns of histones or non-histone proteins could be detected. The fact that the amount of the nuclear proteins remains relatively constant during cell maturation in spite of the pronounced decrease in the rate of their synthesis suggests that the rate of turn-over of these proteins decreases significantly as the maturation of granulocytic leukocytes proceeds.

Significance to Cancer Research: (Objective 2, Approach 3) Understanding of normal and leukemic granulocyte maturation depends upon clarification of the molecular processes whereby the cell controls the appearance and disappearance of specific components. Such information has never been obtained at the biochemical level in a systematic fashion similar to that achieved in morphological studies of normal and leukemic granulocytes. Any biochemical differences in the maturation processes observed between normal and leukemic leukocytes will be exploited for purposes of developing techniques, presently unavailable, for the early diagnosis of leukemia and for improved treatment, especially with regard to the possibility of reversing the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia.

Proposed Course of Research: The rate of production of granulocytic leukocytes in the bone marrow is regulated by a feedback control mechanism which responds to the rate of destruction of these cells in the peripheral tissues of the body. Feedback information could be of a positive (stimulator) or negative (inhibitor) type transmitted by humoral and/or cellular factors, but at present the nature of these factors is unknown. We are using the response of guinea pig bone marrow granulocyte precursors to inflammatory stimuli as a model for studying the nature of the feedback factors involved in the regulation of granulocyte production. The humoral hypothesis for regulation will be examined by studying the effect of sera from normal and inflammatory guinea pigs on the synthesis of granule-specific granulocyte precursor proteins by intact cells in vitro. This approach is analogous to the use of hemoglobin as a marker for erythrocyte production in assays for erythropoietin. Unlike erythrocytes, which contain only one major specific protein,

granulocytes contain many potential marker proteins in their granules and it is conceivable that humoral regulators might affect the synthesis of some granule proteins more than others. In our preliminary studies, therefore, we plan to use the radioactive assay of granule proteins in slices of electrophoretic gels described previously as a screening technique for humoral regulators in sera. If such regulators are detected by this method, then more specific immunological assays for the specific proteins affected by these sera will be developed.

Publications:

Evans, W. H. and Mage, M. G.: Development of surface antigen during maturation of bone marrow neutrophil granulocytes. Exp. Hematol., in press, 1977.

Appendix - NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Collaborating Contract Scientist: Dr. C. K. Hsu
Frederick Cancer Research Center
Frederick, Maryland

Major Findings:

A transplantable granulocytic leukemia in strain 13 guinea pigs has been developed in collaboration with Dr. Hsu. He has carried out extensive macroscopic and microscopic examinations of the leukemic animals at autopsy and has determined that this leukemia is similar to the chronic myelogenous form in humans. The leukemic granulocytes in the blood and marrow of the guinea pig characteristically show the complete range of cell maturity and the leukemic cells infiltrating the spleen, liver, and other body organs are exclusively granulocytes and their precursors. Experiments are in progress to determine whether the abundant virus-like particles found in these cells are of significance in the etiology of this granulocytic leukemia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 375-15 LB

PERIOD COVERED: July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Homogeneity and Structure of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. A. Peterson	Head, Protein Chemistry Section	LB NCI
OTHER:	A. R. Torres	Research Associate	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Protein Chemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Current efforts are directed toward the development of displacement chromatography for the fractionation of macromolecules and particles of biological interest, employing polyanions and polycations differing in molecular size and charge density as displacers.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their function and structure.

Methods Employed: A variety of polyanions and polycations differing in molecular size, charge density, and chemical composition are prepared by appropriate reactions. Most of these are ionic derivatives of low molecular weight dextrans but other types are being explored. They are evaluated as displacing agents, using test protein mixtures, on hydrophilic ion exchangers. Effluent fractions are examined by electrophoresis in slabs of polyacrylamide gel and by appropriate assay.

Major Findings: The capacity of a DEAE-cellulose column for binding carboxymethyldextrans (CM-dextrans) in the immobilized range has been found to be, on a mass basis, directly proportional to their equivalent weights, i.e., inversely proportional to their content of carboxymethyl groups and, consequently, inversely proportional to their affinity for the column. This finding, which is sharply in contrast with the adsorption behavior of proteins, is of great importance in the design of chromatographic separation experiments for it establishes the shape of the fully developed displacement train. It not only permits the selection of appropriate relative concentrations for successive sections of the continuum of displacers used in the separation of complex protein mixtures such as serum, but it also promises to provide a simple means of recognizing, on the basis of a preliminary small scale experiment, the particular range of carboxymethyl group content that will bracket, in affinity, a given protein component so as to expedite focusing the separation on that component in a simpler, large scale separation.

All of the adsorbable CM-dextrans that were studied displaced essentially the same amount of counter ion during saturation of the column, indicating a high degree of configurational accommodation by both adsorbent and CM-dextran to the spacing of charges on each and the existence of a large compartment of the total charges on the adsorbent that was accessible to CM-dextrans of 20,000 MW as well as to those of 10,000 MW.

As expected, the number of bonds formed by ion exchange per molecule of adsorbed CM-dextran, calculated from the counter ion displaced in these experiments, increased with higher carboxymethyl group content and reached values of 18 and 42 for the most highly substituted CM-dextrans of 10,000 and 20,000 MW, respectively, under the conditions employed.

Relatively simple protein mixtures consisting of commercially purified (but still impure) proteins have been used, with CM-dextrans having restricted ranges of moderate and high affinity, to study the mechanism of displacement chromatography on DEAE-cellulose. The shapes of the major protein bands in the emerging displacement trains were found to conform

with those predicted by displacement theory. Ovalbumin and β-lactoglobulin A were easily separated by this procedure, and β-lactoglobulin A was completely separated from β-lactoglobulin B.

The use of CM-dextran in an adsorption method for concentrating proteins has been explored. A dilute protein solution (10 mg in 50 ml) was pumped into a 150 µl column of DEAE-cellulose, and the concentrated band of adsorbed protein was then displaced sharply with a 1% solution of a high-affinity CM-dextran. Ovalbumin, β-lactoglobulin A, and ceruloplasmin were concentrated 50-fold by this procedure, which avoided contamination with high concentrations of salt, a problem accompanying the use of conventional adsorption concentration procedures.

In an analogous way, a solution of goat γ-globulin was concentrated on a small column of CM-cellulose and was then sharply eluted with 1% CM-dextran. In this case the CM-dextran acted as an eluting agent instead of a displacer because it had no affinity for the CM-cellulose; but, as in the displacement procedure, its use instead of salt permitted immediate electrophoresis of the concentrate. Further exploration of the eluting ability of highly substituted CM-dextran has shown it to be effective in gradient chromatography of ribonuclease A and goat γ-globulins on CM-cellulose.

Significance to Cancer Research: (Objective 2, Approach 3). Displacement chromatography of proteins, nucleic acids, etc., promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The displacers, unlike salt, do not interfere with electrophoresis, and protein fractions obtained by displacement can be directly subjected to electrophoresis or rechromatography without dialysis or concentration. Thus the examination of complex mixtures by a combination of chromatography and electrophoresis on a semi-micro scale is greatly expedited. The absence of band-spreading and the retention of the full capacity of the column throughout the process offer additional advantages over elution chromatography.

Moreover, since migration is induced by displacers under conditions of salt and pH in which the migrating molecules are otherwise strongly adsorbed, the possibility arises of chromatographing substances that are "irreversibly" adsorbed in conventional procedures. Also, theoretical considerations strongly suggest that many of the enzymes that become labile when purified can be protected from inactivation or loss by substances such as the CM-dextrans if the metal-sequestering properties of the system are taken into account.

Proposed Course of Research: The method, as it develops, will be applied to the isolation of a serum factor (or factors) that mediates bone marrow response to experimentally induced inflammation in the guinea pig. Its possible application in the fractionation of cell populations and subcellular organelles will be explored, using appropriate adsorbent matrices.

Also, a stabilizing effect of the CM-dextrans will be looked for, using enzymes that have previously been difficult to purify because of their high lability.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 5202-10 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Isolation, Fractionation, and Charcterization of Native Nucleoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	O. Wesley McBride	Senior Surgeon	LB NCI
OTHER:	Raghbir S. Athwal	Visiting Fellow	LB NCI
	Anne S. Olsen	NIH Postdoctoral Fellow	LB NCI

COOPERATING UNITS (if any)

Laboratory of Immunology, Bureau of Biologics, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop methods for gene mapping in eukaryotic cells. Gene transfer is detected by isolation of colonies of biologically transformed cells in selective medium after incubation of isolated metaphase chromosomes with mutant recipient cells deficient in HPRT or TK. The gene product is further characterized as chromosomal donor species. The method currently may be most useful for determining close gene linkages. Nucleic acid hybridization is employed to determine the size of the transferred chromosome fragment (transgenome). Current estimates indicate that about 0.05 to 0.1% of the donor genome is present in a transcriptionally active form in transgenotes.

PHS-6040
(Rev. 10-76)

Objectives: Biological transformation of cells with isolated and fractionated mammalian metaphase chromosomes as a means of genetic mapping.

Methods Employed: Tissue culture procedures involving propagation of mammalian cells in monolayer and suspension cultures, plating in selective media, cloning, labeling with radioisotopes, selection of cell mutants, and metaphase arrest for chromosome isolation. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation (including unit gravity procedures), and evaluation of chromosomal DNA molecular weight by zonal density gradient sedimentation. Gene transfer to mammalian cells via uptake of purified metaphase chromosomes and isolation of biologically transformed colonies in selective media. Isolation, quantitation, and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Isolation and biochemical and karyotypic analysis of somatic cell hybrids following interspecific cell fusion induced by polyethylene glycol 1000. Karyological analysis of cells by fluorescence, phase, and bright-field photomicroscopy. Fixation and staining of cells and chromosomes with fluorochromes for flow microfluorometric analysis of DNA content. Isolation of DNA and nucleic acid hybridization techniques.

Major Findings: The following facts have previously been demonstrated in this laboratory and confirmed in other laboratories:

1. The stable transfer of functional chromosomal donor genes to recipient cells occurs following the uptake of isolated metaphase chromosomes by mammalian cells *in vitro*.

2. The size of the transferred chromosome fragment (transgenome) is smaller than an intact chromosome.

3. The stable cotransfer of very closely linked genes (thymidine kinase and galactokinase) frequently occurs by this process.

It has also been shown that functional genes can be serially transferred by intermediate steps of metaphase chromosome isolation and incubation with recipient cells, indicating that stabilization of the transgenome probably involves its integration into the DNA of the recipient cell.

Human chromosomal donor species of DNA (transgenome) has been isolated from a clonal line of mouse cells containing the transferred human hprt gene (transgenote) in collaboration with Dr. Gurmit Aulakh and this provides an indication of the size of the transgenome. Radiochemically labeled human DNA was hybridized with RNA from the transgenote and it was subsequently hybridized with mouse RNA to remove transcribed DNA sequences homologous with the recipient species. This labeled DNA probe was evaluated by its reassociation in the presence of driver DNA from various sources including mouse (recipient), human (donor) and

transgenote. The human DNA transcribed in this transgenote represents 0.05-0.1% of the human genome. If only 5% of the DNA is transcribed at any time, this suggests that the transgenome represents about 1-2% of the human genome or 40-80% of an intact human X-chromosome. This is a surprisingly large value and seemingly inconsistent with the failure to detect any of three different donor non-selected, X-chromosomal protein markers in this transgenote. It could be explained if multiple copies of the transgenome or multiple donor chromosome fragments are present in the transgenote or if there is no regulatory control for transcription of the transgenome.

The human gene for thymidine kinase has been transferred to mouse TK^- cells by chromosome-mediated gene transfer (CMGT) and several independent clones have been isolated. The human hprt gene has been transferred to Chinese hamster cells and to chloramphenicol resistant mouse cells by CMGT. Biochemical and karyological study of these clones has been partially completed and subclones containing the stabilized (integrated) transgenome are being isolated.

Primary Chinese hamster fibroblasts have been fused with mouse TK^- cells and hybrid clones were isolated. Human-mouse interspecific hybrids were also obtained. Segregants are being isolated containing only the human X-chromosome or human chromosome 17 (containing TK locus) in a mouse background or a single intact hamster chromosome bearing the TK and galactokinase genes. These various transgenotes and reduced hybrid cell lines in combination with our other transgenote lines provide the biological reagents required to further investigate the sites of integration, and size, of the transgenome.

Human hypoxanthine phosphoribosyl transferase (HPRT) has been purified by Dr. Anne Olsen and a goat has been immunized with this enzyme. The goat anti-human HPRT will be a valuable reagent with many potential applications. It will provide a means for rapid identification of the human species of this enzyme in transgenotes as well as a method for purification of HPRT from transgenotes for two-dimensional electrophoresis.

Proposed Course of Research:

Nucleic acid hybridization studies are in progress and the most promising approach involves the covalent attachment of mercury (as reported by Dale and Ward) to ^3H-labeled DNA probes. The conditions for mercuration of DNA to various extents and those for separation of mercurated from non-mercurated DNA on sulfhydryl-agarose columns have been established. The required transgenote and hybrid cell lines are now available to evaluate the following parameters:

1. Size of the transgenome and variation in size among various transgenotes.

2. Variation in transferred DNA sequences (i.e. where did the original fragment start and end) among transgenotes with the same selected marker.

3. Whether a single chromosome fragment or multiple fragments are integrated into the DNA of transgenotes.

4. Evaluation of the number of copies of the transgenome in cells prior to its integration.

5. Whether the size of the transgenome decreases during stabilization (integration).

6. Change in the transgenome size accompanying serial chromosome transfer events.

7. Assessment of possible clustering of families of repetitive DNA in small regions of the transgenome.

Integration sites of the transgenome will be evaluated by several independent approaches. In situ hybridization methods will be developed to quickly and unambiguously identify the species of origin of individual chromosomes in hybrids and transgenotes.

Significance to Cancer Research: (Objective 2, Approach 3) Metaphase chromosome isolation and fractionation provides the most promising approach for the fractionation of mammalian genes and can provide material for the investigation of control elements associated with specific sets of genes. The biological expression of these chromosomes introduced into recipient cells provides a method for mapping of mammalian genes, investigating differences between genetic elements in normal and neoplastic cells, and looking for sites of integration of oncogenic viruses into the mammalian genome. Chromosome-mediated gene transfer also provides a method for cloning relatively small fractions of a eukaryotic genome in an interspecies background. This method should also afford insight relating to control and integration of eukaryotic genes.

Publications:

McBride, O.W., and Athwal, R.S.: Genetic analysis by chromosome-mediated gene transfer. In Vitro 12: 777-786, 1976.

Athwal, R.S., and McBride, O.W.: Serial transfer of a human gene to rodent cells by sequential chromosome-mediated gene transfer. Proc. Natl. Acad. Sci. USA, in press.

McBride, O.W., and Athwal, R.S.: Chromosome-mediated gene transfer with resultant expression and integration of the transferred genes in eukaryotic cells. In Genetic Interaction and Gene Transfer. Brookhaven Symp. Biol. 29, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 5203-09 LB

PERIOD COVERED: July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunochemical and Physical Purifications of Immunocytes and Their Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. G. Mage	Immunochemist	LB NCI
OTHER:	T. L. Rothstein	Staff Fellow	LB NCI
	L. L. McHugh	Biologist	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Protein Chemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is the development of cell separation methods for the specific isolation of immune cells, particularly for varieties of antigen-reactive cells involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Antigen-reactive lymphocytes (ARC) are obtained from the spleens of normal mice and from mice immunized with allogeneic tumor cells and normal cells. Spleen cell suspensions containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. The nonadherent cells are tested for depletion of cytotoxic effector activity by measurement of ^{51}Cr release from target cells. Graft-versus-host (GVH) activity is measured by the Simonsen spleen weight assay in neonatal mice. The mixed lymphocyte culture is used to test for stimulation of ARC by alloantigen in vitro, and for the generation of cytotoxic effector cells from their precursors.

PHS-6040
(Rev. 10-76)

Objectives: The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigen-reactive cells involved in various cellular immune reaction to alloantigens, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules.

Methods Employed: Antigen-reactive cells (ARC) are obtained from the spleens of normal mice or from mice immunized with allogeneic tumor cells and normal cells. Spleen cell suspensions containing ARC are incubated on foam columns or on petri plates coated with allogeneic or syngeneic target cells. Graft-versus-host (GVH) activity of non-adherent spleen cells is measured by the Simonsen spleen weight assay in neonatal F_1 mice. Cytotoxic activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction (MLR) and by generation of cytotoxic effector cells in vitro.

Major Findings

1. We have further characterized the mouse spleen cells without surface immunoglobulin (Ig^- cells) that we have preparatively separated by incubation on petri plates precoated with specifically purified anti-immunoglobulin. Such Ig^- populations retain GVH and MLR activity. They respond to the T cell mitogens Concanavalin A and PHA, and they generate cytotoxic effector cells in vitro. An unexpected finding (in collaboration with Dr. James Mond, I/LI, is that these Ig^- populations can generate antibody plaque-forming cells in vitro, i.e., they probably contain the precursors of B cells that have not yet produced detectable surface Ig.

2. We have further developed a general method of cell separation, based on the binding of antibody-coated cells to anti-immunoglobulin coated surfaces. Using this technique, we have separated Ig^- from Ig^+ spleen cells and in collaboration with Dr. Warren Evans, CLB, have separated mature neutrophils and their precursors from other cell types present in guinea pig bone marrow.

3. We have prepared a guinea pig antibody to mouse cytotoxic effector cells and their precursors. This antibody, which appears to have a minimal effect on several other cellular immune reactions, is being used to help delineate the subtypes of T cells that participate in the GVH and other immune reactions.

4. We have previously shown that a fraction of the ARC that produce a GVH reaction do not bind to allogeneic cells in vitro. In collaboration with Dr. Gideon Goldstein (Sloan Kettering, NYC) we have looked to see if the thymic hormone Thymopoietin can induce differentiation of such cells to develop antigen-binding capacity, so far without success.

Significance to Cancer Research: (Objective 2, Approach 1) These studies on the specific separation of antigen-reactive cells relate to the mechanisms of cell-mediated tumor rejection. Knowledge of the antigen-binding characteristics of immune cells may help in development of specific purifications of immune lymphocytes with antitumor reactivities and may aid in understanding the variation in tumor susceptibility of human beings and make possible control of immune mechanisms of tumor surveillance and rejection.

The GVH reaction is an iatrogenic complication of tumor therapy regimens that include bone-marrow transplantation. A specific separation of GVH-antigen reactive cells might aid in avoiding this complication.

Proposed Course of Research:

We plan to (1) characterize the Ig^- precursors of antibody-forming cells with respect to their differentiation; (2) continue to develop and improve the general cell separation methodology based on the binding of Ig-coated cells to anti-Ig-coated surfaces; (3) attempt to use this methodology, in conjunction with appropriate allo and hetero antisera, to separate various T-cell subtypes; and (4) study separated T-cell subtypes with respect to their contributions to *in vivo* immune phenomena such as GVH reaction, and graft and tumor rejection.

Publications:

Mage, M. G., McHugh, L. L., and Rothstein, T. L.: Mouse lymphocytes with and without surface immunoglobulin: Preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. J. Immunol. Methods, 15: 47-56, 1977.

Evans, W. H., and Mage, M. G.: Development of surface antigen during maturation of bone marrow neutrophil granulocytes. Exp. Hematol. in press 1977.

SUMMARY STATEMENT
LABORATORY OF MOLECULAR BIOLOGY
DCBD, NCI
July 1, 1976 through September 30, 1977

The Laboratory of Molecular Biology expanded last year when space and personnel from the former Laboratory of Biology were transferred to it. These new resources have permitted the hiring of three new senior scientists: Dr. Susan Gottesman, Dr. Michael Gottesman, and Dr. Sue Wickner.

The research efforts in the Laboratory are now divided into six broad areas:

1. The biochemical basis of malignant transformation with particular emphasis currently on the role of cyclic AMP and cell surface protein (CSP) in this process.

2. Characterization of the major proteins that have a role in the movement, shape and adhesion of cultured cells and identification and characterization of additional molecules that regulate shape and movement.

3. Regulation of gene activity with particular emphasis on the identification of molecules that activate genes and the determination of the structure and function of the regulatory regions of genes.

4. Studies on the biochemistry and genetics of the recombination of the E. coli chromosome with the λ chromosome, and studies on the mechanism of synthesis of various bacteriophage DNAs by E. coli enzymes.

5. Investigation of how the thyroid cell forms, stores and releases its hormone and the elucidation of factors that control its growth, differentiation and transformation into a malignant cell.

6. Investigations on mammary tumor virus and other oncornaviruses and elucidation of how these viruses are synthesized and how they change normal cells into cancer cells.

Part I. The Biochemical Basis of Malignant Transformation.

Last year K. Yamada and I. Pastan observed that the readdition of Cell Surface Protein (CSP) to many types of transformed cells restored their appearance, surface morphology and alignment to normal by increasing adhesion of these cells to the substratum and to each other. K. Olden and K. Yamada have investigated why RSV-transformed CEF have less CSP and found that this is mainly due to a decreased synthesis rate. At the same time S. Adams and others in the laboratory have isolated RNA from normal and RSV-transformed CEF and determined that the levels of translatable mRNA for CSP and collagen (also known to be reduced

in RSV-transformed cells) is diminished. This suggests that the product of the transforming region of RNA tumor viruses act at the gene level to alter cellular behavior.

However the loss of CSP does not account for the abnormal growth and enhanced metabolism of RSV-transformed cells since its readdition does not affect these parameters. J. Pouyssegur and I. Pastan have isolated a mutant of Balb 3T3 cells defective in glycoprotein synthesis that has the morphologic features of many transformed cells but is not a tumor cell. Studies with this cell line by M. Willingham and others have shown that substances that increase cell to substratum adhesion restore the morphology of this cell line (and other rounded cells) to normal without affecting the growth properties of such cells. M. Willingham has also confirmed by scanning electron microscopy, a finding previously made by dark field microscopy, that the surfaces of highly agglutinable cells are covered by microvilli.

From RSV-CEF R. Shiu has isolated a 78K dalton protein previously thought to be specifically increased in transformation. He has found that the major increase in this protein and another of 95K daltons in RSV transformed cells is mainly secondary to glucose starvation. The same protein is induced in normal cells by glucose starvation. The function of these proteins is as yet unknown.

G. S. Johnson and F. Cabral observed that the nicotinic acid analogue, picolinic acid, arrests normal cells in the G1 phase of the cell cycle and many transformed cells in G2. Picolinic acid treatment also potentiates the cellular rise in cyclic AMP upon PGE_1 activation. The biochemical basis of this arrest is under study.

W. Anderson has continued his studies on the alterations in adenylate cyclase activity in growing and transformed cells. He has found that the mutant cell AD6 that has a major decrease in its content of the carbohydrate portions of glycoproteins and glycolipids responds normally to PGE_1 and catechol amines but has a decreased response to cholera toxin. This suggests that the former hormones do not have glycoprotein receptors. Anderson has isolated a factor from serum that inhibits the GTP functions of the adenylate cyclase enzyme. The mode of action of this factor is under study.

Part II. Characterization of the Proteins and Factors that Control Cell Shape Movement and Adhesion.

The actin binding protein filamin has been purified from chicken gizzard by P. Davies and guinea pig vas deferens by D. Wallach. It is a phosphoprotein and a substrate for cyclic AMP dependent protein kinase but not for cyclic GMP dependent kinase. P. Davies has isolated a fragment of filamin that retains actin binding properties and like filamin inhibits actin activation of myosin ATPase. To date the role of phosphorylation is not established.

I. Klein has identified a microtubule associated protein fraction in fibroblastic cells. This protein(s) which is found in both the soluble and membrane fractions of the cell is a phosphoprotein. The protein is being further characterized by P. Bechtel.

L. Moore has shown that the internal membranes of fibroblasts are able to transport calcium. The major increase in the activity of this pump occurs when cells crowd together. This pump could lower cytoplasmic Ca^{++} levels in cells and alter cell shape and/or movement in confluent cells.

Michael Gottesman and F. Cabral have begun to isolate mutants of CHO cells defective in cell membrane functions in order to study the genetics and biochemistry of the cell surface.

K. Yamada has obtained sufficient quantities of CSP for its characterization by physical methods. Studies are under way with D. Schlessinger at Harvard Medical School and H. Edelhoch at NIAMDD. Further with Y. Schlessinger, E. Elson, W. Webb and G. Hammes at Cornell the mobility of CSP on the surface of cells and its effect on the modulation of the mobility of other cell proteins has been examined. CSP moves much slower than intrinsic membrane proteins and does not modulate the mobility of intrinsic proteins.

Part III. Regulation of Gene Activity and Structure of Regulatory Loci.

B. de Crombrugghe, R. Di Lauro and S. Adhya have shown that the regulation of the transcription of the gal operon is at 2 overlapping promoters (P1 and P2). Cyclic AMP and CRP activate only P1 and inhibit P2. The gal repressor protein inhibits P1. This allows E. coli to make sufficient galactose metabolites for cell wall biosynthesis when glucose is the carbon source and cyclic AMP levels are low, yet derepress gal enzyme synthesis when galactose becomes the sole carbon source. The nucleotide sequence of the promoter region has been determined in these studies.

S. Adhya, A. Das, D. Court and Max Gottesman have isolated mutants defective in termination factor rho that are conditional lethal for growth. Characterization of these mutants has revealed that rho and RNA polymerase interact for termination of transcription and that the rho mutation has a pleiotropic effect.

D. Court has identified a λ gene whose function affects the permeability of the host's (E. Coli) membrane and reduces retention of cyclic AMP. This mutation also affects the stability of phage RNA perhaps through a membrane interaction.

M. Rosenberg has investigated the nature of the DNA encoded stop signals of various λ genes which are either dependent on or independent of the termination factor rho. He has found that all of the transcripts have 3' terminal sequence heterogenity (mainly 1-5 additional adenylate residues) not encoded in the genome. Further he has evidence that rho factor results in a structural change in the nucleotide seuqence with which RNA molecules can terminate. In collaboration with M. Singer (Lab. Biochemistry) he has determined the sequence of DNA fragments containing monkey DNA sequences from defective SV40 molecules.

Part IV. Recombination and DNA Synthesis

S. Gottesman has been investigating the mechanism of site-specific recombination of bacteriophage λ and shown that the substrate for integration of λ is a covalent negatively-twisted circle. This also may be an intermediate in the excisive reaction.

S. Wickner has been investigating the pathway by which single-stranded circular DNAs are converted to double-stranded DNA by E. coli proteins. She has shown that there are three pathways which share a common mechanism of DNA elongation but use separate mechanisms of priming the template DNA.

Part V. Thyroid Cell Function

S. Wollman and J. Zeligs have continued their investigations of the mechanism of endocytosis in the thyroid and shown that in the hyperplastic thyroid pseudopod formation may be induced by contact with another pseudopod. Wollman has also continued his studies on thyroid development and hyperplasia. These have been supplemented by the development of lines of thyroid cells that grow in tissue culture.

Part IV. Mammory Tumor Virus and Other Oncornoviruses.

G. Smith has gathered evidence that the A particles found in mouse mammary tumors may be precursors for MMTV nucleoprotein cores. G. Vlahakis has continued his studies on the genetic and non-genetic factors that are involved in mouse mammary tumorigenesis. K. Stromberg has been investigating human chornocarcinoma cells to understand the factors regulating the behavior of this cancer. He has found that methotrexate increases the synthesis of chorionic gonadotropin by these cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 8000-07 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Regulation of Gene Activity by cAMP and cGMP

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, LMB	LMB NCI
Other:	S. Adhya, Geneticist	LMB NCI
	D. Schlessinger, (Harvard University)	
	V. Machia, Univ. Naples, Italy	

COOPERATING UNITS (if any)
University of Naples, Naples Italy
Harvard University

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER *

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of cyclic AMP Receptor Protein is being determined by protein sequencing techniques. The possible role of cyclic GMP in E. coli is being investigated by genetic and biochemical techniques.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8000-07 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Regulation of Gene Activity by cAMP and cGMP

Previous Serial Number: ZO1 CBD 8000-06 LMBGY

Principal Investigator: Ira Pastan

Other Investigators: S. Adhya, D. Schlessinger, and V. Macchia

Cooperating Units: Harvard University
University of Naples, Naples, Italy

Man Years: July 1, 1976 through September 30, 1977

Total:	.2
Professional:	.2
Others:	0

Project Description:

Objectives: Cyclic AMP is required for the synthesis of a variety of enzymes in E. coli, most of which are inducible. Cyclic AMP acts at a transcriptional level. Our objective is to determine how cyclic AMP, cyclic AMP receptor protein (CRP) and other factors control gene activity.

Methods Employed: Cyclic AMP Receptor (CRP) has been purified in large amounts. The structure is being determined by protein sequencing methods.

Major Findings: Studies on the sequence of CRP are being continued. A large insoluble fragment that has caused sequencing difficulties previously has been identified as the amino terminal fragment. The sequence of this fragment is nearing completion.

Significance to Biomedical Research and the Program of the Institute: National Cancer Plan Objective 2, Approach 3. As we understand in detail how the expression of bacterial genes is controlled we will be able to understand and then control the expression of the genes of animal cells and modify the behavior of cancer cells.

Proposed Course: To complete the sequence of CRP and determine which region interacts with DNA at promoter sites.

Publications:

Pastan, I., and Adhya, S.: Cyclic adenosine 5'-monophosphate in Escherichia coli. Bacteriol. Rev. 40: 527-551, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 8001-07 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Cyclic AMP in the Regulation of Growth and Morphology of Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira Pastan, Chief, LMB	LMB NCI
Others:	Peter Bechtel, Expert	LMB NCI
	Peter Davies, Surgeon	LMB NCI
	Irwin Klein, Surgeon	LMB NCI
	Leon Moore, Staff Fellow	LMB NCI
	David Wallach, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

Clinical Endocrinology Branch, National Institute Arthritis, Metabolism and Digestive Diseases, National Eye Institute.

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	5.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The adenylate cyclase and guanylate cyclase of cultured cells have been further characterized. Prostaglandin E1 activates a.c. in a highly cooperative manner. The calcium transport activity of the internal membranes of fibroblasts has been found to increase as cells crowd together and fall when they are dispersed. Two phosphoproteins, filamin and microtubule - associated proteins have been purified and characterized. The former binds to actin, the latter to tubulin.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8001-07 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Role of Cyclic AMP in the Regulation of Growth and Morphology of Transformed and Normal Fibroblasts

Previous Serial Number: ZO1 CBD 8001-06 LMBGY

Principal Investigator: Ira Pastan

Other Investigators: Peter Bechtel, Peter Davies, Irwin Klein, Leon Moore, David Wallach

Cooperating Units: Clinical Endocrinology Branch, National Institute Arthritis, Metabolism and Digestive Diseases; National Eye Institute

Man Years: July 1, 1976 through September 30, 1977

Total:	5.5
Professional:	5.5
Other:	1.0

Project Description:

Objectives: To understand the role of cyclic AMP in malignant transformation and how cyclic AMP regulates the growth, morphology, and other properties of cultured fibroblastic cells.

Methods Employed: Cell culture and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules.

Major Findings: Adenylate Cyclase-P. Davies has extensively characterized the ability of prostaglandin E_1 to activate fibroblast adenylate cyclase. Together with Zech and Berman (NIAMDD) a model describing this activation has been developed in which there is a high degree of cooperativity in PGE_1's interaction with the receptor.

I. Klein has attempted to alter the cholesterol content of the plasma membrane by incubating cells with cholesterol-containing liposomes. In some cells this results in a fall in adenylate cyclase (a.c.) activity indicating this lipid could have a physiological role in regulating the activity of a.c.

Guanylate Cyclase: The mechanism by which guanylate cyclase activity is increased in vivo is obscure, although Ca^{++} has been implicated. D. Wallach has shown that low levels of Ca^{++} activate guanylate cyclase in the presence of Mg^{++}; this type of activation may account for how cyclic GMP levels are increased in intact cells.

Calcium-To determine how intracellular calcium levels are controlled in fibroblasts L. Moore has characterized the microsomal calcium uptake system. The system requires Mg^{++} and ATP like the sarcoplasmic reticulum calcium pump of muscle. The activity of the pump is low in sparse 3T3 cells, rises 8-fold as the cells crowd together, and promptly falls when cells are plated at low density. Although most transformed cells are like their parent normal cells, a few transformed cell lines fail to raise their Ca^{++} pump at high density. A high calcium pump activity in crowded cells probably results in low cytoplasmic calicum levels.

Filamin (actin binding protein)-P. Davies has shown that filamin from chicken gizzard and mouse fibroblasts is a phosphoprotein and is a substrate for cyclic AMP dependent protein kinase. To elucidate how phosphorylation might control the activity of filamin, the protein has been characterized by physical-chemical methods and shown to be a large asymmetric flexible molecule composed of multiple domains of organized structure connected by segments of a flexible polypeptide chain. Gizzard filamin binds to F-actin, inhibits actin activation of myosin ATPase and forms a gel. To date phosphorylation of filamin has no effect on its physical properties or ability to inhibit actomyosin ATPase.

D. Wallach has purified filamin from guinea pig vas deferens, a source of mammalian smooth muscle whose contraction is regulated by various hormones. The guinea pig protein resembles gizzard filamin in its molecular weight, amino acid composition and ability to bind actin. It is also immunologically related. Cyclic AMP but not cyclic GMP increases its phosphorylation in broken cell extracts. Cyclic GMP stimulates the phosphorylation of a membrane protein of about the same molecular weight as filamin. These results suggest a direct effect of cyclic AMP on myofilaments and of cyclic GMP on membrane function in smooth muscle.

P. Bechtel has prepared cyclic AMP dependent protein kinase used in studies on filamin phosphorylation. He has also used this protein to phosphorylate microtubule associated proteins to explore their role in the control of cell shape.

I. Klein has investigated whether fibroblasts have microtubule associated proteins (MAPs) similar to those of brain. He has used brain tubulin to polymerize MAPs from fibroblast extracts and found that fibroblasts have a MAP-like protein with a molecular weight between that of the two major MAPs of brain. Fibroblast MAP is a phosphoprotein and a substrate for cyclic AMP dependent protein kinase.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approaches 1, 2 and 5; Objective 4, Approach 2; Objective 6, Approach 3. Various aspects of this work will

lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Proposed Course: 1. Gather further data on cyclic nucleotide levels in cultured cells and how these levels are controlled. 2. Identify physiologically important molecules that regulate guanylate cyclase activity. 3. To determine the role of calcium in the regulation of cell shape by identifying proteins whose activity is controlled by calcium. 4. Further characterized proteins involved in the regulation of shape of cultured cells, with emphasis on phosphoproteins.

Publications:

Nesbitt, J., Anderson, W. B., Miller, Z., Pastan, I., Russell, T. and Gospodarowicz, D.: Guanylate cyclase and cyclic guanosine 3':5'-monophosphate phosphodiesterase activities and cyclic guanosine 3':5'-monophosphate levels in normal and transformed fibroblasts in culture. J. Biol. Chem. 251: 2344-2352, 1976.

Thomopoulos, P., Roth, J., Lovelace, E., and Pastan, I.: Insulin receptors in normal and transformed fibroblasts: Relationship to growth and transformation. Cell 8: 417-423, 1976.

Shizuta, Y., Davies, P., Olden, K., and Pastan, I.: Diminished content of plasma membrane-associated myosin in transformed fibroblasts. Nature 261: 414-415, 1976.

Wallach, D., and Pastan, I.: Stimulation of guanylate cyclase of fibroblasts by free fatty acids. J. Biol. Chem. 251: 5802-5809, 1976.

Ostlund, R. E., and Pastan, I.: The purification and quantitation of myosin from cultured cells. Biochim. Biophys. Acta, 453: 37-47, 1976.

Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I., and Lewis, M. S.: Purification and properties of filamin, an actin binding protein from chicken gizzard. J. Biol. Chem. 251: 6562-6567, 1976.

Wallach, D., and Pastan, I.: Stimulation of membranous guanylate cyclase by concentrations of calcium that are in the physiological range. Biochem. Biophys. Res. Commun. 72: 859-865, 1976.

Moore, L., and Pastan, I.: Regulation of intracellular calcium in chick embryo fibroblast: Calcium uptake by the microsomal fraction. J. Cell. Physiol., 91: 289-296.

Davies, P., Shizuta, Y., Olden, K., Gallo, M., and Pastan, I.: Phosphorylation of filamin and other proteins in cultured fibroblasts. Biochem. Biophys. Res. Commun., 74: 300-307, 1977.

Pastan, I., Willingham, M., Yamada, K., Pouyssegur, J., and Davies, P.: Biochemical basis of the morphologic phenotype of transformed cells. In B. R. Brinkley and Keith R. Porter (Eds.): International Cell Biology, 1976-1977.

Thomopoulos, P., Kosmakos, F. C., Pastan, I., and Lovelace, E.: Cyclic AMP increases the concentration of insulin receptors in cultured fibroblasts and lymphocytes, (in press) 1977.

Davies, P., Bechtel, P., and Pastan, I.: Filamin inhibits actin activation of heavy meromyosin ATPase, (in press) 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8712-02 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira H. Pastan, Chief, LMB	LMB NCI
Others:	Robert Shiu, Guest Worker	LMB NCI
	Jacques Pouyssegur, Visiting Fellow	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A mutant of Balb 3T3 cells was isolated that has the morphologic phenotype of transformed cells but is not transformed. Its transformed appearance is due to its low adhesion which in turn is due to a defect in glycoprotein synthesis. A 78,000 dalton membrane has been isolated from RSV-transformed CEF and found to be present in glucose-starved normal cells.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8712-02 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

Previous Serial Number: ZO1 CBD 8210-01 LMBGY

Principal Investigator: Ira H. Pastan

Other Investigators: Robert Shiu and Jacques Pouyssegur

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	2.2
Professional:	1.2
Other:	1.0

Project Description:

Objectives: To identify the membrane proteins that participate in cell adhesion, cell movement and regulate cell metabolism and to determine if these are altered in malignant transformation.

Methods: Cell culture; preparation and analysis of membrane proteins and their effects on cell behavior; isolation of mutants with defective membrane proteins.

Major Findings: J. Pouyssegur has isolated a mutant of Balb 3T3 cells that has the morphologic phenotype of many transformed cells but has normal growth control. This indicates that low adhesion, high agglutinability by plant lectins, round shape and the presence of microvilli are not sufficient for abnormal cell growth.

R. Shiu has isolated a 78,000 MW protein from CEF transformed by the Schmidt-Ruppin Strain of RSV. This protein as well as one with a 95,000 MW is increased in transformed cells but is also increased by glucose starvation of normal cells. Thus their presence is not specific for transformation. The possible function of these proteins is under investigation.

Significance to Biomedical Research and the Program of the Institute: The invasive properties of tumor cells may be due to altered membrane proteins. Identification and isolation these proteins may suggest new methods of cancer treatment. Objective 3, approach 3.5.

Proposed Course: To isolate additional mutants defective in surface properties. To identify other surface proteins important in the control of cell shape and movement.

Olden, K., Willingham, M., and Pastan, I.: Cell surface myosin in cultured fibroblasts. Cell 8: 383-390, 1976.

Yamada, K. M., and Pastan, I.: Cell surface protein and neoplastic transformation. Trends in Biochemical Sciences 1: 222-224, 1976.

Yamada, K. M., Ohanian, S. H., and Pastan, I.: Cell surface protein decreases microvilli and ruffles on transformed mouse and chick cells. Cell 9: 241-245, 1976.

Yamada, K. M., and Pastan, I.: The relationship between cell surface protein and glucose and α-aminoisobutyrate transport in transformed chick and mouse cells. Supplement issue J. Cell. Physiol., 89: 827-830, 1976.

Pouyssegur, J. and Pastan, I.: Mutants of mouse fibroblasts altered in the synthesis of cell surface glycoproteins preliminary evidence for a defect in the acetylation of glucosamine-6-phosphate. J. Biol. Chem., 252: 1639-1646, 1977.

Pouyssegur, J., Willingham, M., and Pastan, I.: Role of cell surface carbohydrates and proteins in cell behavior: Studies on the biochemical reversion of an N-acetyl glucosamine deficient fibroblast mutant. Proc. Natl. Acad. Sci. USA, 74: 243-247.

Willingham, M. C., Yamada, K. M., Yamada, S.S., Pouyssegur, J., and Pastan, I.: Microfilament bundles and cell shape are related to adhesiveness to substratum and are dissociable from growth control in cultured fibroblasts. Cell, 10: 375-380, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 8011-03 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Yamada, Investigator	LMB NCI
Other:	Kenneth Olden, Expert	LMB NCI
	Ira Pastan, Chief, LMB	LMB NCI

COOPERATING UNITS (if any)

Gene Regulation Section

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institute of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	2.1	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A major cell surface glycoprotein (CSP) that is involved in cellular adhesion is depleted after malignant transformation of fibroblasts in vitro. Reconstitution of purified, exogenous CSP on the surfaces of transformed cells partially restores a normal phenotype, including a fibroblastic cell morphology and alignment of cells, a smooth cell surface architecture with few microvilli and ruffles, and a restoration of intracellular microfilament bundles. CSP does not decrease the rate of glucose or amino acid transport.

The decrease in CSP after transformation is due primarily to decreased biosynthesis, although losses from increased rates of protein turnover also contribute. The decreased biosynthesis appears to result from decreased amounts of messenger RNA for CSP.

Our objectives will be to determine the composition and structure of CSP, and the mechanisms by which it helps to maintain normal cell behavior.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8011-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein

Previous Serial Number: ZO1 CBD 8011-02 LMBGY

Principal Investigator: Kenneth M. Yamada

Other Investigators: Kenneth Olden
Ira Pastan

Cooperating Units: Gene Regulation Section, LMB

Man Years: July 1, 1976 through September 30, 1977

Total:	2.9
Professional:	2.1
Other:	0.8

Project Description:

Objectives: A major cell surface protein (CSP) is depleted after neoplastic transformation of fibroblasts in vitro. Our objectives are to determine CSP's biochemical structure and its role in cell behavior.

Methods Employed: CSP is isolated from chick embryo fibroblasts using urea, then further purified by salt fractionation and column chromatography at alkaline pH. Monospecificity of anti-CSP antisera is evaluated using a new histochemical procedure developed to detect antigens separated by electrophoresis in SDS polyacrylamide gels. Antigens are visualized with diaminobenzidine using peroxidase coupled to antibody. Concanavalin A receptors are detected by a modification of this technique. CSP is quantitated by a double immunoprecipitation procedure in which the protein is labeled with ^{14}C leucine, solubilized at pH 11, diluted and neutralized for immunoprecipitation with affinity-purified anti-CSP. The immunoprecipitate is analyzed on SDS gels. The rate of processing and transport of CSP to the cell surface is determined by rate at which newly synthesized CSP labeled with ^{14}C leucine becomes trypsin-sensitive as it becomes exposed on the cell surface.

Major Findings: We had previously shown that isolated CSP is an adhesive molecule that increases cell-to-cell and cell-to-substratum adhesiveness. Reconstituting isolated, purified CSP on the surfaces of transformed cells resulted in partial reversion of altered cell morphology

and the abnormal patterns of overlapping and alignment characteristic of transformed cells. We now find that CSP also restores a more normal cell surface architecture by suppressing microvilli and marginal ruffles. CSP treatment can also restore the intracellular cytoskeleton of microfilament bundles. However, exogenous CSP does not restore a normal rate of transport of either glucose or the amino acid analog aminoisobutyrate.

Using a modification of a new procedure developed to localize antigens in SDS polyacrylamide gels, we find that CSP is the major receptor for the lectin concanavalin A in chick fibroblasts.

We used quantitative immunoprecipitation to study the mechanisms of the decrease in CSP after transformation of chick fibroblasts by Rous sarcoma virus. The major mechanism for each of 4 strains of Rous sarcoma virus is a 3-6 fold decrease in the rate of biosynthesis of CSP. After synthesis, quantities of CSP are further decreased by an increased rate of protein turnover. In collaborative experiments, the amount of messenger RNA for CSP that could be translated in an in vitro translation system is also found to be decreased 5-fold after transformation.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 5. Neoplastic cells in culture generally display faulty growth control and increased transport of glucose and amino acids, altered morphology, decreased adhesion, loss of monolayering and alignment, increased cell surface microvilli and ruffles, and loss of intracellular microfilament bundles. Our studies indicate that CSP plays a major role in the maintenance of these characteristics, with the exception of growth control and nutrient transport. The loss of CSP after transformation, and the abnormalities in cell behavior due to its loss, appear to be due primarily to a decrease in the amount of CSP messenger RNA, which results in a marked decrease in the rate of CSP biosynthesis.

Preoposed Course: To determine CSP's distribution, mobility, composition, and structure, and to explore the role of carbohydrates and disulfide groups in determining its biological activity.

Publications:

Yamada, K.M., Yamada, S.S., and Pastan, I.: Cell Surface Protein Partially Restores Morphology, Adhesiveness, and Contact Inhibition of Movement to Transformed Fibroblasts. Proc. Natl. Acad. Sci. USA 73: 1217-1221, 1976.

Yamada, K.M., Ohanian, S.H., and Pastan, I.: Cell Surface Protein Decreases Microvilli and Ruffles on Transformed Mouse and Chick Cells. Cell 9: 241-245, 1976.

Yamada, K.M., and Pastan, I.: The Relationship between Cell Surface Protein and Glucose and α-Aminoisobutyrate Transport in Transformed Chick and Mouse Cells. J. Cell. Physiol. 98: 827-830, 1976.

Yamada, K.M., and Pastan, I.: Cell Surface Protein and Neoplastic Transformation. Trends in Biochemical Sciences 1: 222-224, 1976.

Olden, K., and Yamada, K.M.: Direct Detection of Antigens in Sodium Dodecyl Sulfate-Polyacrylamide Gels. Analyt. Biochem. 78: 483-490, 1977.

Willingham, M.C., Yamada, K.M., Yamada, S.S., Pouyssegur, J., and Pastan, I.: Microfilament Bundles and Cell Shape are Related to Adhesiveness to Substratum and are Dissocialbe from Growth Control in Cultured Fibroblasts. Cell 10: 373-380, 1977.

Olden, K., and Yamada, K.M.: Mechanism of the Decrease in the Major Cell Surface Protein of Chick Embryo Fibroblasts after Transformation. Cell (in press) 1977.

Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K.M., de Crombrugghe, B., and Pastan, I. Reduction in Translatable Messenger RNA for Cell Surface Protein and for Collagen Precursors and Increase in Functional Messenger RNA for Two Membrane Proteins in Rous Sarcoma Virus-Transformed Chick Embryo Fibroblasts. Proc. Natl. Acad. Sci. USA (in press) 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8705-01 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Genetic and Biochemical Analysis of Cell Surface Function.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.M. Gottesman	Senior Investigator	LMB	NCI
Other:	F. Cabral	Senior Staff Fellow	LMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing the Chinese Hamster Ovary (CHO) fibroblast grown in vitro to study the genetics and biochemistry of the cell surface. Mutants of this cell line defective in adhesion have been isolated and will be characterized with respect to their cell surface proteins. CHO cells can cause tumors in appropriate host animals and we will determine the effect of specific cell surface alterations on tumor formation.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 8705-01 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Genetic and Biochemical Analysis of Cell Surface Function

Previous Serial No.: None

Principal Investigators: Michael M. Gottesman

Other Investigators: Fernando Cabral (beginning December 1976)

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total	1.5
Professional:	1.5
Other:	0.0

Projective Description:

Objectives: To determine by genetic manipulation the cell surface proteins needed for cell adhesion and cell-cell interaction.

Methods: Cell culture; isolation of mutants with altered cell surface functions; electrophoretic analysis and preparation of plasma membranes.

Major Findings: We have isolated mutants of CHO cells defective in their ability to adhere to substrate. To date, all of these mutants have altered morphology, but retain normal growth properties. In some, the addition of exogenous cAMP or drugs which increase endogenous cAMP repair the morphologic and adhesive defect. Biochemical characterization of these mutants is in progress.

Significance for Cancer Research and the Program of the Institute: CHO cells will cause tumors in appropriate hosts. The identification of mutant CHO cells with specific defects in cell surface functions will enable us to determine which of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at neutralizing those cell surface functions required for tumor formation can be devised. Objective 3, Approach 3.5.

Proposed Course: To isolate many classes of mutants with defective cell surface function and determine specific protein alterations in these mutants.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 8010-03 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Morphologic Mechanisms of Motility and Malignant Transformation in Cultured Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mark C. Willingham, Senior Investigator	LMB NCI
Other:	Ira H. Pastan, Chief, LMB	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.50	2.50	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Transformation of cultured fibroblasts produces drastic changes in cell shape, adhesiveness, surface morphology, and agglutination by plant lectins. The changes that occur in the distribution of microfilamentous contractile proteins after transformation were found to be the result of changes in cell shape as a result of primary changes in cell to substratum adhesiveness, and not directly related to changes in growth properties. Changes in lectin agglutination after transformation have been linked to changes in surface morphology (microvilli) which are a direct result of cell shape changes. The most significant change in growth properties, anchorage dependence, has been narrowed to an effect on the exit from the G_1 phase of the cell cycle. A new method of ultrastructural immunocytochemical localization has been developed which will help in the understanding of the distribution and function of cell proteins.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8010-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: The Morphologic Mechanisms of Motility and Malignant Transformation in Cultured Cells

Previous Serial Number: ZO1 CBD 8010-02 LMBGY

Principal Investigator: Mark C. Willingham

Other Investigators: Ira H. Pastan, Chief, LMB

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	2.50
Professional:	2.50
Others:	0

Project Description:

Objectives: To investigate the mechanisms that control the expression of properties of transformed cells in culture.

Methods Employed: Cell culture, specialized light and electron microscopic morphologic and immunohistochemical methods, radiolabeling of cultured cell surfaces and internal macromolecules.

Major Findings: Cultured fibroblasts often undergo drastic changes in shape, motility, and growth following malignant transformation. We have studied the mechanisms behind these changes through the alterations in shape, adhesiveness, surface morphology, agglutination by plant lectins, the distribution of contractile protein elements, and growth properties. We have found that growth properties can be separated from these other characteristics.

Through the use of immunohistochemistry and specially selected transformed cells and mutants of normal cells, we have found that adhesiveness to substratum is the primary property regulating all the other morphologic properties. Through manipulation of adhesion, both normal and transformed cells can be made to show the "morphologic phenotype" (shape, villous surface, agglutination by lectins, and bundled contractile protein elements) of either normal or transformed cells without affecting their growth.

Through the use of scanning electron microscopy, we have also reconfirmed the direct relation between surface villous morphology and agglutination by plant lectins previously demonstrated with dark field microscopy.

Of the growth properties altered after transformation, anchorage-dependent growth in suspension seems to be the most direct in vitro measure of tumorigenicity in vivo. We have studied the behavior of normal cells in suspension and their growth requirement for anchorage. We have found that normal cells survive well in suspension, but are synchronized in the G_1 phase of the cell cycle. We have investigated the rapid onset of DNA synthesis when the cells are supplied with a substratum, in order to understand this control mechanism missing after transformation.

The ability to localize specific molecules in cells greatly enhances the understanding of how these molecules function. Immunohistochemical localization provides a unique, highly specific method for this task. Unfortunately, ultrastructural immunolocalization previously had been severly limited due to technical difficulties. Pre-embedding localization, while having major advantages in convenience and antigenic survival, has been restricted due to: 1) the tight matrix produced by fixatives needed for good morphologic preservation, preventing penetration of antibody molecules, 2) the impermeability of membranous structures, and 3) the nonspecific background binding of antibodies due to aldehyde fixatives. We have developed a new method for immunocytochemical localization at the ultrastructural level which allows pre-embedding labelling in the presence of good morphologic preservation in cultured cells. With this method, we are studying the distribution of protein elements involved in cell motility to which we have developed antibodies: actin, myosin, filamin, tubulin, microtubule-associated proteins, and cell surface protein (CSP). The pattern of localization of these molecules should give greater insight into their interrelationships and function than that derived from light microscopy.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3.

It appears that transformation of cultured cells in vitro is closely linked to the ability to form malignant tumors in vivo. By understanding the specific properties and their mechanisms in vitro that are essential for these altered growth properties, one might be able to control the growth and spread of tumors in vivo.

Proposed Course: We will study the mechanisms of anchorage dependence and motility in cultured fibroblasts in order to understand the most basic growth difference in vitro between normal and transformed cells. In addition, we will attempt to clarify further the functional mechanisms of contractile proteins in these cells and their role in anchorage and cell motility, both of which may be intimately involved in the mechanisms of metastasis and the lack of anchorage-dependence of growth in transformed cells.

Publications:

Willingham, M.C.: Quantitation of Agglutination with an Automatic Particle Counter. H. Bittiger and H. P. Schnebli, (Eds.): Concanavalin A as a Tool. John Wiley and Sons, Ltd., 1976, pp. 285-292.

Willingham, M.C.: Cyclic AMP and Cultured Cell Behavior. Int. Rev. of Cytology 44: 319-363, 1976.

Olden, K.; Willingham, M.C., and Pastan: Cell Surface Myosin in Cultured Fibroblasts. Cell 8: 383-390, 1976.

Pouyssegur, J.; Willingham, M.C., and Pastan, I.: Role of Cell Surface Carbohydrates and Proteins in Cell Behavior: Studies on the Biochemical Reversion of an N-acetylglucosamine-deficient Fibroblast Mutant. Proc. Natl. Acad. Sci. USA 74: 243-247, 1977.

Willingham, M.C.; Yamada, K.M.; Yamada, S.S.; Pouyssegur, J.; and Pastan, I.: Microfilament Bundles and Cell Shape are Related to Adhesiveness to Substratum and are Dissociable from Growth Control in Cultured Fibroblasts. Cell 10: 375-380, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 8012-03 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Adenylate Cyclase Activity of Transformed and Normal Fibroblasts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Wayne B. Anderson, Research Chemist LMB NCI
Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.0	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous studies have shown that adenylate cyclase activity of fibroblasts in culture is altered following viral transformation and during various stages of cell growth. Thus, it is of interest to elucidate how the activity of adenylate cyclase might be altered and regulated by both intra-and extra cellular agents and events. We have found that cyclase activity in the AD6 mutant of Balb 3T3 cells is not changed and retains its hormonal responsiveness even though cholera toxin activation is decreased. Since AD6 cells are defective in glycolipid (ganglioside) and glycoprotein synthesis, these results suggest that changes in membrane structure other than modification of the carbohydrate moieties are responsible for the loss of growth control and the altered properties of adenylate cyclase noted with transformed cells. Serum factor(s) may play a significant role in the regulation of adenylate cyclase. We have isolated a factor from calf serum which selectively inhibits the GTP functions of this complex enzyme system. In other studies we have found that the cyclase activity of isolated membrane preparations is markedly enhanced by low concentrations of trypsin or certain other proteolytic enzymes. The mode of action and physiological significance of these regulatory effectors remains to be established.

Serial No. ZO1 CB 8012-03 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Adenylate Cyclase Activity of Transformed and Normal Fibroblasts.

Previous Serial No.: ZO1 CBD 8012-02 LMBGY

Principal Investigator: Wayne B. Anderson

Other Investigators: None

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	2.4
Professional:	1.0
Other:	1.4

Project Description:

Objective: To investigate how adenylate cyclase activity is regulated in normal fibroblasts and how the activity of this enzyme is altered with transformation by RNA tumor viruses.

Methods Employed: Cell culture and standard biochemical analysis of adenylate cyclase activity.

Major Findings: Studies have been devoted to determining how adenylate cyclase activity might be regulated in normal fibroblasts growing in culture. A prime source of possible extracellular modulators of fibroblastic adenylate cyclase is the serum required for the growth of these cells. We have isolated a 60,000 M.W. factor from serum (purified 150 fold) which selectively inhibits GTP and GTP hormone stimulated cyclase. This fraction apparently constitutes a complex of inhibitor binding protein as further fractionation yields a low M.W. inhibitor which is also specific for the GTP functions of the enzyme. Unfortunately, the activity of the low M.W. form of the inhibitor is readily lost so further purification and chemical identification of this potentially interesting factor has been difficult.

Another finding is that the adenylate cyclase activity in crude membrane preparations of confluent NRK and Balb 3T3 fibroblasts is markedly increased by low concentrations (10^{-8} - 10^{-7} M) of trypsin present in the reaction mixture. However, the cyclase activity from subconfluent

NRK cells and from chick embryo fibroblasts is not significantly enhanced by trypsin. Other proteolytic enzymes, including plasmin, elastase, and papain also activated adenylate cyclase, while thrombin and leucine amino peptidase exerted only an inhibitory effect. Trypsin activation is most pronounced (6 fold increase) when A.C. is measured in the presence of 10^{-4}M GTP. Trypsin also increased basal, GTP + PGE_1-stimulated and GTP + epinephrine-stimulated cyclase activity by 2.5 to 3 fold, while NaF stimulated activity was enhanced only 1.4 fold. Pronounced trypsin activation of adenylate cyclase is dependent upon the presence of the metal chelator EGTA.

We have utilized a mutant of mouse fibroblasts (AD6) which is defective in the N-acetylation of glucosamine-6 phosphate (and thus in the biosynthesis of complex gangliosides) to study the effect of altered ganglioside composition of the plasma membrane on adenylate cyclase activities. In support of decreased ganglioside composition is the finding that cholera toxin does not readily activate AD6 adenylate cyclase. However the incorporation of exogenous ganglioside GM_1, or the growth of AD6 cells in N-acetyl glucosamine to overcome the biochemical defect, leads to a marked increase in choleragen activation of cyclase. Even though the modified ganglioside pattern of AD6 is similar to that found with many transformed cells, the activity and hormonal responsiveness of adenylate cyclase is identical to that of the wild-type parent Balb/c 3T3; the enzyme from Kirsten sarcoma virus or SV40 virus transformed cells exhibits low responsiveness to the addition of hormones. These results do not support the suggestion that an alteration in ganglioside biosynthesis in transformed cells leads to altered adenylate cyclase activities and to altered growth properties.

Significance for Biomedical Research and the Program of the Institute: Cyclic AMP has an important role in regulating many properties of normal fibroblasts, as well as numerous biological processes in other cell types. Thus, the regulation of adenylate cyclase is of considerable interest. Further, in studying the properties of the normal enzyme we can learn something of the means by which the enzyme has been altered by viral transformation. If it is possible to correct the defective enzyme of transformed cells, this would result in elevated cyclic AMP levels which in turn could revert some of the abnormal properties of cancer cells toward normal.

Proposed Course: We will continue to elucidate the regulatory properties of the fibroblast adenylate cyclase system. Of concern are means to stabilize, isolate, and characterize the serum inhibitor and to determine its effect on the enzymes involved in cyclic nucleotide metabolism with isolated membrane preparations and with intact cells. The means by which proteolytic enzymes are able to activate adenylate cyclase will also be studied. With SDS slab gel electrophoresis we hope to be able to identify which membrane protein or proteins are modified during the activation process. It will also be of interest to determine if an endogenous membrane protease exists which is able to enhance cyclase activity. Additional studies will be carried out on the ability of

certain growth promoters, such as somatomedins, insulin, and epidermal growth factor, to influence the intracellular concentration of cyclic AMP and to alter adenylate cyclase activity. Finally, an attempt will be made to develop an *in vitro* system to study the mechanism for hormonal desensitization of the cyclase system.

Publications:

Anderson, W.B., and Jaworski, C.J.: Modulation of adenylate cyclase activity of fibroblasts by free fatty acids and phospholipids. Arch. Biochem. Biophys., in press, 1977.

Anderson, W.B., and Jaworski, C.J.: Adenylate cyclase activity of normal and transformed fibroblasts in culture. Proceedings of the Third Dicennial Review Conference, in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8709-02 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George S. Johnson, Research Chemist	LMB NCI
Other:	Jose A. Fernandez-Pol, Guest Worker	LMB NCI
	John Collins, Guest Worker	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NRK cells treated with picolinic acid are reversibly arrested in G_1 and are very responsive to agents which elevate cyclic AMP levels. Other pyridine derivatives are ineffective. NAD levels are not changed, but nicotinamide partially overcomes the growth inhbition. The growth of transformed cells is also reversibly arrested but the block point is dependent upon the nature of the transforming agent. The results point to a role for NAD^+ in growth control.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8709-02 LMB
1. Laboratory of Molecular Biology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Relationship of Carbohydrate and NAD^+ Metabolism to Cell Growth.

Previous Serial Number: ZO1 CBD 8209-01 LMBGY

Principal Investigator: George S. Johnson

Other Investigator: Jose A. Fernandez-Pol
John Collins

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	2.0
Professional:	2.0
Others:	1.0

Project Description:

Objectives: The addition of the pyridine derivatives, picolinic acid, to the medium of cultured cells has pronounced effects on cell growth and cyclic AMP metabolism. Evidence suggests that the actions may require NAD^+ dependent functions. The object of this project is to delineate the mechanism of action of pyridine derivatives with emphasis on NAD^+ functions and metabolism.

Methods Employed: Standard tissue culture techniques will be used to grow cells. Thin layer chromatography and fluorescent analysis will be used to analyze the metabolic components.

Major Findings: Effect of pyridine derivatives on cell growth: Picolinic Acid (2-pyridine carboxylic acid) reversibly arrests the growth of cultured cells. Untransformed cells are blocked in the G_1 phase of the mitotic cycle. The block point in transformed cells is dependent upon the nature of the transforming agent but independent of the origin of the cells. Since picolinic acid is an analogue of nicotinic acid, the effects on NAD^+ metabolism were tested. Picolinic acid did not change the amount of NAD^+ in the cells, however, nicotinamide partially overcame the growth inhibition. Other pyridine derivatives did not reversibly arrest growth or prevent the effects of picolinic acid. These results suggest that picolinic acid interacts with a specific growth control mechanism which may involve NAD^+, and that this control mechanism is altered by different transforming agents in different manners.

Effect of pyridine derivatives on cyclic AMP metabolism: Cyclic AMP has been implicated as an important element in growth control; thus we reasoned that picolinic acid could be working through alterations in cyclic AMP metabolism. Basal cyclic AMP levels are unchanged in NRK cells arrested in G_1 by picolinic acid; however, three agents which activate adenylate cyclase, Prostaglandin E_1, isoproterenol, and cholera toxin, are 3-5 times more potent in elevating cyclic AMP levels in these cells than in control cells.

Significance for Cancer Research and the Program of the Institute: By understanding how growth and metabolism are regulated and by understanding differences in this regulation between normal and transformed cells we may learn to control the growth of certain cancer cells.

Proposed Course: Further studies will be done to understand the molecular basis for the effects of picolinic acid on cultured cells. Emphasis will be placed on understanding how nicotinamide overcomes these effects. Hormone binding assays and ATP and GTP analysis will be done to understand how picolinic acid alters cyclic AMP metabolism. These studies may be useful in understanding the mechanism of action of "hormone-like" agents in the culture medium which regulate cell growth.

Publications:

Schwartz, J.P. and Johnson, G.S.: Metabolic Effects of Glucose Deprivation and of Various Sugars in Normal and Transformed Fibroblast Cell Lines. Archives Biochem. Biophys. 173: 237-245.

Johnson, G.S.: Cyclic Nucleotides in Normal and Transformed Fibroblasts. W. E. Criss, T. Ono, and J. Sabine, (Eds): Modified Cellular and Molecular Control in Neoplasia. Raven Press, N.Y., 1976, pp. 153-160.

Johnson, G.S., and Fernandez-Pol, J.A.: NRK Cells Synchronized in G_1 by Picolinic Acid are Super-Sensitive to Prostaglandin E_1 Stimulation. FEBS Letters 74: 201-204, 1977.

Fernandez-Pol, J.A., Bono, V.A., and Johnson, G.S.: Control of Growth by Picolinic Acid: Differential Response of Normal and Transformed Cells. Proc. Natl. Acad. Sci. USA, in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8700-05 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies on the Regulation of Gene Expression in Bacterial and Animal Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. de Crombrugghe	Head, Gene Regulation Section	LMB	NCI
Other:	Bruce Howard	Research Associate	LMB	NCI
	Roberto DiLauro	Visiting Fellow	LMB	NCI
	Sherrill Adams	Staff Fellow	LMB	NCI
	Mark Sobel	Research Associate	LMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The control of the galactose operon in E. coli is studied as a model for gene regulation. We have demonstrated that the regulation of this operon is exerted by two overlapping promoters P1 and P2. The cyclic AMP receptor (CRP) and cyclic AMP (cAMP) activate only P1; gal repressor inhibits transcription from P1 only. P2 is repressed by CRP and cAMP and is probably subject to another regulatory mechanism. We have determined the nucleotide sequence of the entire regulatory region for the wild type gal operon and the base changes in several mutants in this region. This operon constitutes an excellent model system for the study of complex genetic regulation. (2) In collaboration with Dr. Ira Pastan, we are studying the changes in cellular gene expression which occur when animal cells are transformed by oncogenic viruses. We have demonstrated that the levels of translatable mRNA for two large extracellular proteins, the Cell Surface Protein and Collagen, are considerably reduced in transformed cells. These two proteins play an important role in the cellular interactions between normal cells. We are preparing DNA probes which will enable us to directly measure the levels, rate of synthesis and half-life of mRNAs for collagen and other transformation sensitive proteins in different lines of normal and transformed cells and in differentiating tissues.

Serial No. Z01 CB 8700-05 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Studies on the Regulation of Gene Expression in Bacterial and Animal Cells.

Previous Serial No.: Z01 CBD 8008-04 LMBGY

Principal Investigator: Benoit de Crombrugghe, Head, Gene Regulation Section

Other Investigators: Bruce Howard, Research Associate; Roberto DiLauro, Visiting Fellow; Sherrill Adams, Staff Fellow; Mark Sobel, Research Associate

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	5.5
Professional:	4.5
Other:	1.0

Project Description:

Objective: To understand the precise molecular mechanisms which control the expression of specific genes in bacterial and animal cells.

Methods Employed: Measurements of RNA and protein synthesis in vivo and in cell-free systems; synthesis and purification of specific DNA fragments and RNA species; nucleotide sequence analysis of DNA and RNA; introduction of segments of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; purification of factors active in regulation of gene expression.

Major Findings: 1) We use the galactose operon of E. coli as a model system to study gene regulation. We have determined the nucleotide sequence of the entire regulatory region for the wild type gal operon and for a number of mutants in this region. We have demonstrated that the regulation of this operon is exerted by two overlapping promoters (P1 and P2) subject to different regulatory mechanisms. The cyclic AMP Receptor (CRP) and cyclic AMP (cAMP) activate only P1, not P2. The gal repressor inhibits transcription only from P1. In the absence of CRP or cAMP transcription initiates only at P2. CRP and cAMP inhibit transcription from P2. The repressor binds at a

site where it selectively blocks transcription from P1 not from P2, by interfering with the interaction between CRP-cAMP, DNA and RNA polymerase. We have analyzed and sequenced two gal promoter mutants. In these mutants the activity of P1 is abolished but P2 is normal. Thus both the stimulation and the repression of gene expression by CRP and cAMP are exemplified by this system. We believe that this complex mode of regulation reflects the dual role of galactose in the cell. Galactose after it is converted to galactose-1-P can serve as a general carbon source and energy source for cellular metabolism; under the form of UDP-galactose it is also a direct precursor for cell wall biosynthesis. The gal repressor protein is present in the cells in very low concentrations. In order to be able to purify the gal repressor we decided to amplify the number of gene copies by introducing the gene for gal repressor in a plasmid. Such plasmid was constructed by in vitro recombination techniques. E. coli harboring this plasmid contains more than 10-fold more gal repressor than normal cells. We extensively purified the gal repressor protein from these cells and investigated its role in vitro.

2) In collaboration with Dr. Ira Pastan, we are studying the changes in cellular gene expression which occur when animal cells are transformed by oncogenic viruses. Our particular interest is in the regulation of the synthesis of two important extracellular proteins which play an important role in the interaction of normal cells with their neighboring cells, Collagen and Cell Surface Protein (CSP). These proteins are present in reduced amounts in transformed cells. We sought to determine the cause for this reduction by translating total RNA from normal and Rous Sarcoma virus transformed chick embryo fibroblasts (CEF) in a cell-free system from wheat germ. We find that transformed CEF have a 5-fold reduction in the level of translatable mRNA for Cell Surface Protein and a 10-fold reduction in translatable mRNA for collagen precursors. In addition increases in functional mRNA are observed in transformed cells for myosin and for two membrane polypeptides with molecular weights of 95,000 and 78,000.

Our efforts have also been directed at obtaining a DNA probe corresponding to a segment of the collagen gene. We have devised a method whereby a specific DNA probe is obtained without complete purification of the corresponding mRNA. The principle of the method is as follows. Using as template a mixture of mRNAs enriched for a specific mRNA (for instance the mRNA for collagen or CRP), an oligo-dT primer and the enzyme reverse transcriptase double stranded (ds) cDNA is synthesized. This ds cDNA is then cleaved by one or more restriction endonucleases and the cleavage products fractionated by polyacrylamide gel electrophoresis. The most prominent fragments, corresponding to the enriched mRNA species, are purified (eventually by recleavage with a second restriction enzyme). A specific fragment is identified as corresponding to a specific protein by translating in vitro the mRNA which hybridizes to the DNA fragment.

Significance for Cancer Research and the Program of the Institute: The E. coli galactose operon is an excellent model for complex genetic regulatory mechanism. We anticipate that similar complex controlling mechanisms regulate gene expression in animal cells. Our other studies using normal and cancerous cells compare the activity of a few specific genes, encoding proteins which are important for the interactions between cells. Such studies aim at establishing the molecular mechanisms which regulate the activity of these genes.

Proposed Course: (1) Prove that the two promoters of the gal operon are activated in vivo under different physiological conditions; define the precise interaction between the controlling proteins and the DNA regulatory segment of the gal operon; purify additional factors which regulate expression of the gal operon. (2) Obtain a DNA probe corresponding to part of the collagen gene; use this probe to measure directly the levels of mRNA for collagen in several cell lines of normal and transformed cells, and in differentiating tissues; obtain a plasmid containing a segment of the collagen gene; obtain a second plasmid containing a larger portion of the collagen gene and possibly its regulatory elements; initiate in vitro and in vivo studies on the regulation of this gene; explore other differences in cellular gene activity in normal and transformed cells and in differentiating tissues.

Publications:

Rosenberg, M., de Crombrugghe, B. and Musso, R.: Determination of Nucleotide Sequences Beyond the Sites of Transcriptional Termination. PNAS 73: 717-721, 1976.

Adhya, S., Gottesman, M., de Crombrugghe, B. and Court, D.: Transcription Termination Regulates Gene Expression. RNA Polymerase, Cold Spring Harbor, N.Y., R. Losick and M. Chamberlin (Eds.), 1976.

Howard, B. and de Crombrugghe, B.: ATPase Activity Required for Termination of Transcription by the E. coli Protein Factor rho. J. Biol. Chem. 251: 2520-2524, 1976.

Di Lauro, R., Musso, R., Rosenberg, M. and de Crombrugghe, B.: DNA Sequence of the Regulatory Region of the Galactose Operon. Federation Proceedings 1976.

Howard, B.H., de Crombrugghe, B., and Rosenberg, M.: Transcription in vitro of Bacteriophage Lambda 4S RNA: Studies on Termination and rho Protein. Nucleic Acids Res. 4: 827-842, 1977.

Musso, R., DiLauro, R., Rosenberg, M. and de Crombrugghe, B.: Nucleotide Sequence of the Operator-Promoter Region of the Galactose Operon of Escherichia coli. PNAS 74: 106-110, 1977.

Sklar, J., Weissman, S., Musso, R., DiLauro, R., and de Crombrugghe, B.: Determination of the Nucleotide Sequence of Part of the Regulatory Region for the Galactose Operon from Escherichia coli. J. Biol. Chem 252: 3538-3547, 1977.

de Crombrugghe, B.: DNA-Dependent Cell-Free Protein Synthesis. In Weissbach, H. (Ed.): Molecular Mechanisms of Protein Biosynthesis. Academic Press, 1977, in press.

Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K.M., de Crombrugghe, B. and Pastan, I.: Levels of Translatable mRNAs for Cell Surface Protein, Collagen Precursors and Two Membrane Proteins are Altered in Rous Sarcoma Virus-Transformed Chick Embryo Fibroblasts. PNAS, 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8006-06 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of Gene Expression in E. coli K12 Bacteriophages λ and P22.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sankar L. Adhya, Geneticist	LMB NCI
	Max E. Gottesman, Head, Biochemical Genetics Section	LMB NCI
	Donald L. Court, Staff Fellow	LMB NCI
Other:	Asis Das, Visiting Fellow	LMB NCI
	Amos Oppenheim, Visiting Associate	LMB NCI
	Oscar Reyes, Visiting Fellow, left on August 28, 1976	LMB NCI
	Sandra Hilliker, Staff Fellow	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics

INSTITUTE AND LOCATION

National Cancer Institute , NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	6.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously suggested that the gal operon of E. coli has two promoters. We have obtained additional evidence showing that (1) both the promoters are inducible by galactose; (2) one promoter is activated by cyclic AMP and its receptor protein, the other becoming active only in the absence of the cyclic nucleotide or the receptor protein; and (3) operator constitutive mutations can affect the expression of either or both the promoters.

We have previously isolated mutants, defective in transcription termination factor Rho, which are conditional lethal for growth. Further characterization of these mutants have revealed that (1) Rho and RNA polymerase interact for transcription termination, consistent with our previously proposed model, and (2) the rho mutation has pleiotropic effect.

We have also extended our study on the antitermination role of gene N of phage λ and P22, the effect of λ development on host cell physiology, and pattern of λ transcripts obtained after infection of several mutant hosts.

Serial No. ZO1 CB 8006-06 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Control of Gene Expression in E. coli K12 Bacteriophages λ and P22.

Previous Serial Number: ZO1 CBD 8006-05 LMBGY

Principal Investigators: Sankar L. Adhya, Max E. Gottesman, Donald L. Court, Asis K. Das and Amos B. Oppenheim

Other Investigators: Oscar Reyes and Sandra Hilliker

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	6.0
Professional:	6.0
Others:	0.0

Project Description:

Objectives: The gal operon of E. coli is normally subjected to both a positive control and a negative control by cyclic AMP and its receptor protein. The operon may also be subjected to a different type of control whereby expression of distal genes is regulated by the amount of transcriptional readthrough at various termination signals located within the operon. Our object is to analyze the control signals--initiation and termination--of the gal operon and identify the regulatory molecules that interact with these signals. We are also studying the expression of genes in temperate bacteriophages λ and P22 and their effect on the transformation of host cell physiology and gene expression.

Mutation in transcription termination factor (rho) gene affected a number of other cellular proteins. We are investigating the possibility whether there is overlap of genetic information in the locus, which controls the Rho protein.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) Based on our previous work, we have proposed a model of transcription termination by Rho. We have now established, consistent with the model, that Rho is involved in transcription termination in vivo and Rho and RNA polymerase interact in vivo for transcription termination.

(2) rho gene in E. coli also controls the synthesis of other proteins e.g. membrane ATPase, UV repair and recombination enzymes, Rep protein, etc. We suggest that either the subunit of Rho is also shared by these enzymes or there is an overlap of genes for these proteins.

(3) Mutation in rho has enabled the antiterminator (N) function of phage λ to overcome stop signals, which are not normally antiterminated by N in the presence of Rho.

(4) The antiterminator element of phage P22 has also been characterized. The action of the element like that of λ, is promoter-specific but the specificity is less stringent.

(5) A gene has been identified whose function affects the permeability of the host's (E. coli) membrane. It reduces retention of cAMP and uptake of macromolecular precursors. At the same time, it has an affect on increasing the stability of its own RNA perhaps through a membrane interaction.

(6) We have analyzed the host functions Rho and RNase III in λ gene expression. We are also analyzing the mRNA processing events in phage development. Phage mutants which partly alleviate the requirement for RNase III processing are being studied.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Object 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e. expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using gal as a model system. This understanding might help to prevent conversions of cells to those capable of forming cancers.

Proposed Course: 1. To determine the complete structure of the gal operon. 2. To identify all the regulatory molecules involved in turning on and off the various genes of the gal operon, phage λ and P22. 3. To understand the biochemistry of the initiation and termination signals of gene expression. 4. To understand the transformations caused by phage growth on its host. 5. To study the structure of rho gene.

Publications:

Adhya, S., Gottesman, M., de Crombrugghe, B. and Court, D.: Transcription termination regulates gene expression. In M. Chamberlin & R. Losick, (Eds.): RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1976, pp. 719-730.

Das, A., Court, D., Adhya, S.: Isolation and characterization of conditional lethal mutants of E. coli defective in transcription termination factor rho. Proc. Natl. Acad. Sci. USA 73: 1959-1963, 1976.

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Pastan, I., and Adhya, S.: Cyclic Adenosine 5'-Monophosphate in Escherichia coli. Bacteriol. Rev. 40: 527-551, 1976.

Echols, H., Court, D. and Green, L.: On the nature of cis-acting regulatory proteins and genetic organization in bacteriophage: the example of gene Q of bacteriophage λ. Genetics, 83: 5-10, 1976.

Dambly, C. Court, D. and Brachet P: On the ability of a polarity suppressor mutation (suA1) to correct N but not Q defects of phage λ. Molec. Gen. Genet. 148: 175-182, 1976.

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Court, D., Adhya, S., Nash, H. and Enquist, L.: The phage λ integration protein (Int) is subject to control by the cII and cIII gene products, In A. Bukhari, J. Shapiro and S. Adhya (eds.): DNA Insertion Elements, Plasmids and Episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, in press.

Shapiro, J., Adhya, S. and Bukhari, A.: Introduction to DNA Insertion Elements, Episomes and Plasmids, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, in press 1977.

Das, A., Court, D. and Adhya, S.: Pleiotropic effect of rho mutation in E. coli in Symposium on "Molecular Basis of Host-Virus Interaction."

Adhya, S., Gottesman, M. and Court, D.: Independence of gene N and Tof functions of bacteriophage λ. J. Mol. Biol., in press, 1977.

Das, A., Gottesman, M., Court, D. and Adhya, S.: Role of Rho in gene expression in E. coli. Eleventh FEBS Meeting, in press 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8701-03 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

An Examination of a Variety of Nucleic Acid Structural and Functional Relationships Involved in the Regulation of Cellular and Viral Gene Expression.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. Rosenberg Senior Investigator LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been investigating nucleic acid structure involved in the control of transcriptional initiation, termination, and post-transcriptional processing and modification of various RNA transcription products. Nucleic acid sequences have been determined in the 3'-terminal regions of a number of bacterial RNA polymerase mediated transcription products synthesized from λ and λ gal transducing phages. We have also examined nucleic acid structure in the "terminator" regions of the DNA template beyond the 3'-ends of several RNA transcription products. We are presently defining the structure surrounding an RNA polymerase stop-signal in bacteriophage λ which is totally dependent on the protein termination factor, rho, for its function. Nucleic acid structure in the regulatory region of the galactose operon of E. coli has also been examined.

In addition, the nucleotide sequence of a region of the bacteriophage T7 genome which lies between two adjacent "early" messenger RNAs has been completed. This intercistronic region contains all of the structural information necessary for specifying the processing event (i.e. cleavage by the host encoded endonuclease RNase III) which matures this polycistronic precursor RNA into individual monocistronic mRNAs.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 8701-03 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: An examination of a variety of nucleic acid structural and functional relationships involved in the regulation of cellular and viral gene expression.

Previous Serial Number: Z01 CBD 8013-02 LMBGY

Principal Investigator: Martin Rosenberg

Other Investigators: C. Brady

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	3.0
Professional:	2.0
Other:	1.0

Project Description:

Objectives: 1) To determine the nucleic acid structure involved in the control of transcriptional termination and post-transcriptional modification of RNA and to understand the functional relationship of this structure to the regulation of these processes.

2) To determine the nucleic acid structure involved in the regulation of gene expression of the galactose operon of E. coli.

3) To examine certain highly reiterated DNA sequences in the monkey genome and their interaction with simian virus 40.

4) To investigate the ability of defined bacterial and phage mRNAs to be efficiently translated in embryotic cell-free systems.

5) To develop new techniques which can be applied to both these studies and to the general examination of nucleic acid structure in other biological systems.

Methods Employed: Purification of specific RNA transcripts synthesized either in vivo or in vitro; nucleic acid sequence analysis of both RNA and DNA; primed extension of RNA transcription products with deoxyribonucleotides; isolation of protein factors involved either in the processes of transcriptional termination or post-transcriptional modification of RNA; isolation of purified

DNA restriction enzymes and their employment in obtaining specific purified fragments of both viral and cellular DNAs.

Major Findings: 1) The nucleic acid primary structure at the termini of the bacteriophage T7 "early" mRNAs synthesized both in vivo and in vitro have been examined. The sequence information obtained supports a model for transcription of the "early" region in which a high molecular weight precursor RNA molecule is post-transcriptionally processed to yield monocistronic messengers. The processing agent is a host specified endonuclease, Ribonuclease III, which is also involved in the post-transcriptional processing of a ribosomal RNA precursor in E. coli. Thus, it was shown that the T7 "early" RNAs are direct products of RNase III cleavage of a large polycistronic precursor molecule.

In addition, we demonstrated that the 3'-termini of the processed RNAs contained sequence heterogeneity arising from the addition of several (up to 5) adenylate residues to the 3'-end. These A additions appear to be a non-template specified, post-transcriptional, post-processing, modification of the RNA.

In an effort to further elucidate the mode of action of RNase III in the processing event, as well as examine the entire structure of an intercistronic region of the phage, we have now isolated and sequenced an RNA fragment which traverses the region between two adjacent "early" T7 genes (thereby spanning an RNase III cleavage site).

Sequence analysis of this fragment indicates that the processing enzyme produces a single endonucleolytic cleavage in this region giving rise directly to the G0.3 and G0.7 mRNAs. In addition, the 3'-terminal adenylic acid residues observed previously on the in vivo T7 early RNA species were not found in these fragments and, therefore, must represent a post-transcriptional, post-processing modification of the RNA.

The secondary structure of this region is also quite characteristic. Two relatively stable base-paired stretches of RNA adjoin the cleavage site in a somewhat symetrical arrangement. These double-stranded regions appear to be required for recognition of the cleavage site by RNase III. The site of scission however is located in a small region of RNA set between the double-stranded regions.

2) The nucleic acid structure of the 3'-terminal regions of a number of RNA transcripts synthesized in vitro from λ and λ gal transducing phages are being examined. These RNAs represent both independently terminated transcription products as well as transcripts requiring the protein factor, Rho, for their termination. Sequence analysis at the 3'-ends of some of these products has indicated:

a) that all of the transcripts examined possess similar degrees of 3'-terminal sequence hetereogeneity which consisted predominately of the addition of 1 to 5 adenylate residues to the 3'-terminus of the transcript and b) that rho factor enhanced termination results in a definite structural change in the nucleotide sequence with which an RNA molecule can terminate.

In addition, we have developed a new methodology for examining the nucleic acid structure in the untranscribed portion of the DNA immediately adjacent to the 3'-terminus of an RNA transcript. This technique has been applied to the determination of sequence information throughout the entire termination region of these transcription products. Little structional homology is apparent in the template DNA beyond the 3'-ends of these transcripts. The lack of homology suggests that this region might not be important to the termination process. Thus, the majority of the encoded termination signal is transcribed into RNA.

More recently we have determined the DNA sequence surrounding and examined termination at a totally rho dependent site (t_{R1}) in bacteriophage λ. The DNA composition at t_{R1} is 75% AT base pairs and termination occurs heterogeneously over four adjacent base pairs. No GC rich or U rich sequence is found at the 3'OH end of the RNA. This sequence is quite different from other transcripts that terminate independently of Rho. However, there is a stable base-paired stem and loop structure near the end of this RNA.

In the absence of Rho factor with normal transcription conditions (i.e. 37° and high triphosphate levels), RNA polymerase pauses several seconds at this site. A mutation, cnc, that interferes with base pairing in the stem and loop, has two effects on transcription: RNA polymerase does not pause in the absence of Rho and transcription does not terminate in the presence of Rho. This suggests that the stem and loop induces RNA polymerase to pause, and that a paused polymerase is important for Rho action.

3) DNA fragments containing monkey DNA sequences have been isolated from defective SV40 genomes that carry host sequences in place of portions of the SV40 genome. The fragments were isolated by restriction endonuclease cleavage and contain segments homologous to sequences in both the highly repetitive and unique (or less repetitive) classes of monkey DNA. The complete nucleotide sequence of one such fragment (151 base pairs) predominantly homologous to the highly reiterated class of monkey DNA was determined using both RNA and DNA sequencing methods. The nucleotide sequence of this homogeneous DNA segment does not contain discernible multiple internal repeating units but only a few short oligonucleotide repeats. The reiteration frequency of the sequence in the monkey genome is greater than 10^6. Digestion of total monkey DNA (from uninfected cells) with endonuclease R HindIII produces relatively large amounts of discrete DNA fragments that contain extensive regions homologous to the fragment isolated from the defective SV40 DNA.

A second fragment, also containing monkey sequences, was isolated from the same defective substituted SV40 genome. The nucleotide sequence of the 33 base pairs of this second fragment that are contiguous to the 151 base pair fragment has also been determined.

The sequences in both fragments are also present in other, independently derived defective substituted SV40 genomes.

More recently, pure DNA fragments containing these repeated sequences have been isolated after restriction endonuclease digestion of total monkey DNA. Comparison of the nucleotide sequences in the DNA segments isolated directly from the monkey genome with those obtained from the substituted SV40 DNA yields information about the organization of highly repeated structures in the monkey genome and the nature of the interaction between the virus and its host.

4) The galactose operon of E. coli is subject to both negative control by gal repressor and positive control by cAMP and its receptor protein (CRP). We have sought to define the nucleotide sequence of the promoter-operator sites responsible for gal regulation. Analysis of transcripts of restriction fragments combined with direct DNA sequencing enabled us to derive the sequence for the gal regulatory region. Cleavage by restriction endonuclease Hin f abolishes cAMP-CRP dependent but not CRP independent transcription. Formation of a cAMP-CRP dependent preinitiation complex prevents cleavage by Hin f. Thus the region upstream of the Hin f site is important for cAMP-CRP stimulation of transcription. This region contains sequence similarities with the CRP recognition site of lac. One gal operator constitutive mutant is resistant to Hin f cleavage suggesting the operator may include at least part of the Hin f site. Further analyses are required to confirm this possibility.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. The regulation of gene expression in both prokaryotic and eukaryotic systems involves numerous and complex structural and functional interactions between nucleic acids and proteins. It is highly probable (and experimental evidence increasingly supports the contention) that many of the nucleic acid fine-structure interactions which occur on the prokaryotic level have basic similarities to analogous type interactions known to occur in higher cells. Our studies involve the determination of the nucleic acid structure involved in the control of the initiation and termination of transcription by RNA polymerase and the post-transcriptional processing of certain RNA species by specific endonucleases (e.g. RNase III) in both bacterial and related viral systems. The elucidation of these transcriptional processes and their regulatory elements will be a basis for understanding similar processes occurring in eukaryotic cells (both normal and transformed) and virus (oncogenic and other).

Proposed Course: To examine the mechanisms by which RNA transcripts are initiated, terminated and subsequently processed during the cellular and viral life cycle and to investigate further the structure-function relationships involved in a variety of nucleic acid-protein interactions.

Publications:

Sogin, M.L., Pace, N.R., Rosenberg, M., and Weissman, S.M.: The Nucleotide Sequence of a 5S Ribosomal RNA Precursor from Bacillus subtilis. J. Biol. Chem. 251 (11): 3480-3488.

Rosenberg, M., de Crombrugghe, B., and Musso, R.: Determination of Nucleotide Sequence Beyond the Sites of Transcriptional Termination. PNAS 73: 717-721, 1976.

Kramer, R., and Rosenberg, M.: The Isolation and Characterization of Bacteriophage T7 mRNA Fragments Containing an RNase III Cleavage Site. Nuc. Acids Res. 3: 2411-2426, 1976.

Segal, S., Garner, M., Singer, M.F. and Rosenberg, M.: In Situ Hybridization of Repetitive Monkey Genome Sequences Isolated from Defective Simian Virus 40 DNA. Cell 9: 247-257, 1976.

Musso, R., DiLauro, R., Rosenberg, M. and de Crombrugghe, B.: Nucleotide Sequence of the Operator-Promoter Region of the Galactose Operon of Escherichia coli. PNAS 74: 106-110, 1977.

Rosenberg, M. and Kramer, R.: Nucleotide Sequence Surrounding A Ribonuclease III Processing Site in Bacteriophage T7 RNA. PNAS 74: 984-988, 1977.

Keene, J.D., Rosenberg, M., and Lazzarini, R.A.: Characterization of the 3' Terminus of RNA Isolated from Vesicular Stomatitus Virus and its Defective Particles. PNAS 74: 1353-1357, 1977.

Howard, B.H., de Crombrugghe, B., and Rosenberg, M.: Transcription In Vitro of Bacteriophage Lambda 4S RNA: Studies on Termination and Rho Protein. Nuc. Acids Res. (1977) In Press.

Musso, R.E.and Rosenberg, M.: Nucleotide Sequences at Two Sites for IS2 DNA Insertion. In Bukhari, W.I. (Ed.): DNA Insertion Elements, Plasmids, and Episomes. Cold Spring Harbor Laboratory, 1977, in press.

Rosenberg, M., Segal, S., Kuff, E.L., and Singer, M.F.: The Nucleotide Sequence of Repetitive Monkey DNA Found in Defective Simian Virus 40. Cell, 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8711-01 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Site-Specific Recombination in Bacteriophage Lambda.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Gottesman, Research Chemist	LMB NCI
	Max Gottesman, Head, Biochemical Genetics Section	LMB NCI
Other:	Patsy Trisler, Bio. Lab. Technician	LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER x

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the biochemistry and genetics of lambda site-specific recombination. An E. coli mutation, rap, which blocks the growth of normal lambda is permissive for lambda variants with deletions at or near the attachment site. We are improving our in vitro site-specific recombination assay with a view to isolating and purifying the components.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8711-01 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Mechanism of Site-Specific Recombination in Bacteriophage Lambda.

Previous Serial Number: None

Principal Investigator: Susan Gottesman, Max Gottesman

Other Investigators: Patsy Trisler

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	2.0
Professional:	1.5
Other:	.5

Project Description:

Objectives: Bacteriophage Lambda inserts into and excises from the E. coli chromsome. This recombination occurs at a unique site in the viral and host chromosomes, and is promoted by several functions. We have previously demonstrated the excision reaction in vitro. Our object is to continue to study the mechanism of phage excision, by purifying and analyzing the components of the in vitro system.

An E. coli mutant, rap, is non-permissive for wild-type lambda, but plates lambda variants with altered attachment sites. We intend to study this phenomenon.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) The substrate for integrative reaction is lambda DNA in the form of a covalent, negatively-twisted circle. Based on in vivo studies we find that a similar topology may be required for the excisive reaction as well. (2) We have found two lambda variants with normal attachment sites which nevertheless grow on rap strains: a) a lambda deleted for a portion of its genome in the b2 region to the left of the attachment site and b) a deletion-mutant missing DNA to the right of the attachment site.

Significance for Cancer Research and the Program of the Institute: It is likely that oncogeneses involves the integration and excision of mammalian viruses. Lambda site-specific recombination is a model system for such interactions.

Proposed Course: Isolation and purification of the enzymes and cofactors required for prophage excision. Identification of these factors with respect to host and viral mutations which eliminate site-specific recombination. To determine why lambda fails to grow on rap strains and how the lambda mutations which allow growth overcome this restriction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8710-02 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
DNA Replication In Vitro Catalyzed by E. Coli Proteins.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sue Wickner, Staff Fellow LMB NCI
Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, National Institutes of Health, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three pathways have been described by which single-stranded circular phage DNAs can be converted to double-stranded DNA in reactions catalyzed by purified E. coli proteins. The three pathways use different mechanisms of priming the template DNA but share a common mechanism of DNA elongation catalyzed by DNA polymerase III, dnaZ protein and DNA elongation factors I and III. In the simplist pathway fd(or M13) DNA synthesis by the DNA elongation components is primed by RNA synthesized by RNA polymerase. In the most complex pathway, ØX174 DNA synthesis requires dnaB, C(D), DNA binding protein, at least three other E. coli proteins (DNA replication factors X, Y and Z) and the same four DNA elongation proteins. A third pathway used G4(or ST-1) DNA, dnaG protein, and DNA binding protein as well as the DNA elongation proteins. The functions of these purified proteins and the mechanism by which they catalyze DNA synthesis are being studied.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8710-02 LMB
1. Laboratory of Molecular Biology
2. Biochemical Genetics Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: DNA Replication In Vitro Catalyzed by E. Coli Proteins

Previous Serial Number: ZO1 AM 33003-01 LMB

Principal Investigator: Sue Wickner

Other Investigators: None

Cooperating Units: None

Man Years: July 1, 1976 through September 30, 1977

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objective: To gain information about basic biochemical mechanisms involved in the process of DNA replication.

Methods Employed: Standard biochemical techniques.

Major Findings:

1. Mechanism of DNA elongation catalyzed by E. coli DNA polymerase III, dnaZ protein and DNA elongation factors I and III. Elongation of a primed, single-stranded DNA template catalyzed by E. coli DNA polymerase III requires dnaZ protein and two other protein factors, DNA elongation factors I and III. The reaction occurs by the following mechanism: (A) dnaZ protein and DNA elongation factor III together catalyze the transfer of DNA elongation factor I to a primed DNA template. This transfer reaction requires ATP or dATP in addition to dnaZ protein, DNA elongation factors I and III, and primed template; it does not require DNA polymerase III. (B) DNA polymerase III binds to the complex of DNA elongation factor I with primed template; it does not bind to primed template which is not complexed with DNA elongation factor I. This binding reaction proceeds in the absence of ATP or dATP as cofactor, dnaZ protein, and DNA elongation factor III and without additional DNA elongation factor I. (C) The complex of DNA polymerase III, DNA elongation factor I and primed template catalyzes DNA synthesis upon the addition of dNTPs.

2. DNA or RNA priming of G4 DNA synthesis by E. coli dnaG protein. E. coli dnaG protein is involved in the initiation of DNA synthesis dependent on G4 or ST-1 single-stranded phage DNAs. The reaction occurs by the following mechanism: (A) dnaG protein binds to specific sites on the DNA in a reaction requiring E. coli DNA binding protein. (B) An oligonucleotide is synthesized in a reaction involving dnaG protein. With G4 DNA this reaction requires ADP specifically, TTP (or UTP) and dGTP (or GTP). (C) Elongation of the oligonucleotide can be catalyzed by DNA polymerase II or III in combination with dnaZ protein and DNA elongation factors I and III, presumably by the mechanism summarized above.

Significance for Cancer Research and the Program of the Institute: The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of (1) mutants defective in DNA synthesis and (2) large amount of bacteria necessary for biochemical studies. It is expected that the understanding that this work is generating about the process of DNA replication in E. coli will shed light on the nature of the same process in animal cells. Perhaps if normal replication were understood, we would be better able to study the abnormalities of cancer cells.

Proposed course: To continue studying biochemical mechanisms involved in DNA synthesis using purified proteins and defined DNA templates.

Publications:

Wickner, S.: Mechanism of DNA elongation catalyzed by E. coli DNA polymerase III, dnaZ protein and DNA elongation factors I and III. Proc. Natl. Acad. Sci. 73: 3511-3515, 1976.

Wickner, S.: DNA or RNA priming of G4 DNA synthesis by E. coli dnaG protein. Proc. Natl. Acad. Sci. (in press) 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8702-16 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Endocytosis in the Thyroid Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.H. Wollman	Head, Cell Organization Section	LMB	NCI
Other:	J.D. Zeligs	Research Associate	LMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Cell Organization Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The typical thyroid epithelial cell can take in colloid from the follicular lumen by macropinocytosis. It can also phagocytose red blood cells. We propose to study the mechanism of these processes by electron microscopy, histochemistry and related techniques.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 8702-16 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Endocytosis in the Thyroid Gland

Previous Serial Number: Z01 CBD 8401-15 LMBCY

Principal Investigator: Seymour H. Wollman

Other Investigators: Joseph D. Zeligs

Man Years:

Total:	1.5
Professional:	1.0
Other:	0.5

Project Description:

Objectives: To determine mechanism of thyroid hormone release, and more generally, endocytotic properties of typical thyroid epithelium.

Methods: We have made electron microscope observations on cytoplasmic projections from the apical end of the epithelial cell into the lumen of the follicle in the hyperplastic thyroid gland.

Major Findings:

Induction of Pseudopods by contact: In the very hyperplastic thyroid gland colloid is largely depleted from the follicle. The distance between cells on opposite sides of the follicle may be as small as 1 μm. In such follicles there is a relatively high incidence of pseudopods in pairs in close contact, one pseudopod from each of 2 cells on opposite sides of the lumen. Occasionally there was just a single pseudopod in contact with the apical surface of a cell from the opposite side of the lumen. At points of contact there was indication of an early stage of pseudopod formation. These observations suggest that pseudopods can be induced in the apical surface of a cell by contact with another pseudopod.

Significance for Cancer Research and the Program of the Institute: This project fits most clearly under Objective 3, Approach 5, having to do with properties of the cell surface.

Proposed Course: Further studies of the induction and nature of pseudopods will be undertaken.

Publications:

Zeligs, J.D. and Wollman, S.H.: Ultrastructure of Blebbing and Phagocytosis of Blebs by Hyperplastic Thyroid Cells *in vivo*. *J. Cell Biol.* 72: 584-594, 1977.

Zeligs, J.D. and Wollman, S.H.: Ultrastructure of Erythrophagocytosis and Red Blood Cell Fission in Thyroid Epithelial Cells *in vivo*. *J. Ultrastruc. Res.* 59: 57-69, 1977.

Zeligs, J.D. and Wollman, S.H.: Pseudopod Behavior in Hyperplastic Thyroid Follicles *in vivo*. *J. Ultrastruc. Res.* (1977) in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8703-25 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Thyroid Hormone Synthesis and Storage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S.H. Wollman Head, Cell Organization Section LMB NCI

Other: None

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has as its objectives to learn about: (1) The mechanism by which the thyroid gland maintains a concentration of iodide elevated above that of the blood, and (2) The mechanism by which the thyroid gland forms and accumulates protein-bound iodine.

The work is largely based upon in vivo studies and makes use of autoradiography, cytochemistry at ultrastructural level, kinetic studies, and inhibitors of steps in the synthesis of thyroid hormones.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8703-25 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 to September 30, 1977

Project Title: Thyroid Hormone Synthesis and Storage

Previous Serial Number: ZO1 CBD 905-24 LMBGY

Principal Investigator: Seymour H. Wollman

Man Years: July 1, 1976 to September 30, 1977

Total:	0.0
Professional:	0.0
Other:	0.0

Project Description:

Objectives: To learn about the mechanism of synthesis and storage of thyroid hormones.

Major Findings: None

Proposed Course: To be resumed in the future.

Significance for Cancer Research and the Program of the Institute: Some of the work reported here may best be put into Objective 3, Approach 5 because they give information on properties of cell surface and cell membranes.

Publications:

Bernstein, L.H. and Wollman, S.H.: A Circumferential Bundle of Microfilaments Associated with Desmosomes Near the Apex of Typical Thyroid Epithelial Cells. J. Ultrastructure Res. 56: 326-330, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8704-25 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Production and Properties of Transplantable Thyroid Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.H. Wollman	Head, Cell Organization Section	LMB	NCI
Other:	J.D. Zeligs	Research Associate	LMB	NCI
	J.P. Herveg	Guest Worker	LMB	NCI
	F.S. Ambesi-Impiombato	Visiting Associate	LMB	NCI
	L. Nitsch	Visiting Fellow	LMB	NCI

COOPERATING UNITS (if any)
S.R. Hilfer, Dept. of Biology, Temple University, Philadelphia, PA. and Laboratory of Cell Biology, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study the thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring during growth, hyperplasia and involution, growth and properties of thyroid cells and thyroid tumor cells in culture.

We are also studying a variety of types of cells just recently recognized to be present in the normal thyroid gland and which may be the cells of origin of some of the different histologic types of thyroid tumors which have been observed in the past.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8704-25 LMB
1. Laboratory of Molecular Biology
2. Cell Organization Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Production and Properties of Transplantable Thyroid Tumors

Previous Serial No.: ZO1 CBD 906-24 LMBGY

Principal Investigator: Seymour H. Wollman

Other Investigators: J.D. Zeligs, S.R. Hilfer, J.P. Herveg, F.S. Ambesi-Impiombato, H. Coon and L. Nitsch

Cooperating Units: Department of Biology, Temple University
Philadelphia, Pennsylvania, and
Laboratory of Cell Biology, DCBD, NCI

Man Years: July 1, 1976 through September 30, 1977

Total:	3.5
Professional:	3.0
Other:	0.5

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution. We are also studying a variety of cells recently recognized to be present in the normal thyroid gland and which may be the cells of origin of some of the different histologic types of thyroid tumors observed in the past. We are also trying to grow normal and neoplastis thyroid epithelium in vitro.

Methods Employed: Origin of cell types in thyroid: Ventral and ultimobranchial outpocketings of the embryonic pharynx were removed prior to their fusion to form the developing thyroid gland. They were implanted separately in the kidney capsule of the adult and allowed to differentiate. Later they were removed and examined by electron microscopy. Hyperplasia: Hyperplasia was produced in rat thyroid gland by feeding of the goitrogen, thiouracil. Various details of the nature of the growth process were examined by electron microscopy. Details included the nature of the miotic process in epithelial and endothelial cells. Tissue culture: Cells were grown in vitro in the presence or in the absence of a fibroblast feeder layer. Stereology of the hyperplastic thyroid: Quantitative changes in the number and volume of epithelial cells, volume of blood vessels and follicular lumens were determined on histologic sections during development of hyperplasia.

Results: Origin of cell types (with Hilfer): Transplants of ultimobranchial outpocketings into the kidney capsule give different results in mouse and rat. In the mouse only 1 type of follicle is found containing C cells as well as other strange cell types. In the rat as reported last year there are two types of follicles, one containing the C cells separated from the other types.

Hyperplasia (with Herveg): After 3 weeks of growth stimulation only a minor part of the increase in thyroid growth comes from an increase in thyroid hormone producing cells. A major part comes from increase in blood and in capsule. The increase in capsule in part involves incorporation of neighboring major blood vessels, nerve and striated muscle. During development of hyperplasia, capillaries in the thyroid enlarge until they contact neighbors and fuse. Almost all epithelial cells come into close approximation to capillaries. The capillary response is restricted to the thyroid. Similar enlargement of capillaries does not occur in the parathyroid gland although it is partially embedded in the thyroid.

Mitosis (with Zeligs): The ultrastructure of the typical thyroid epithelial cell and of the capillary endothelial cell have been examined at various stages of mitosis. We have followed the duplication of the centriole and aspects of the division of the cells including the formation of the contractile ring and midbody and the fate of the midbody. Since neighboring cells remain attached to the mitotic cell they undergo deformation as the mitotic cell cleaves.

Tissue culture of thyroid cells (with S. Ambesi and L. Nitsch): Thyroid epithelial cells have been grown in conditioned medium and cloned. The growth pattern of the epithelial cells differs depending on whether they are a single cell type or in mixed culture with thyroid fibroblasts.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. The properties of cells and of the tumors derived from them frequently depend upon the embryologic origin of the cells. This project will allow us to understand better the properties of certain tumors. The study of thyroid hyperplasia may give insight into conditions existing in the gland at the time that neoplastic transformation occurs.

Proposed Course: I plan to continue the above studies of hyperplasia.

Publications:

Zeligs, J.D. and Wollman, S.H.: Microhemorrhage in the Hyperplastic Thyroid Gland of the Rat. Am. J. Pathology 85: 317-332, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 8706-06 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mouse Mammary Tumor Virus - Host Cell Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.H. Smith	Research Biologist	LMB	NCI

COOPERATING UNITS (if any)

Dr. Barbara Vonderhaar; LPP, DCBD, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolation of intracytoplasmic A particles from primary and transplanted mouse mammary tumors. Biochemical, immunologic, ultrastructural, and biologic characterization of the purified particles and comparison of those results with those obtained with similarly purified mouse mammary tumor virus (MMTV). A particle proteins were isolated from SDS-polyacrylamide gels, three MMTV antigenic determinants P^{27}, P^{14}, P^{10} were demonstrated to be associated with A particle structural polypeptides by immunodiffusion. A precursor-product relationship betweeen A particles and MMTV nucleoprotein cores is becoming apparent.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 8706-06 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through June 30, 1977

Project Title: Mouse Mammary Tumor Virus - Host Cell Interaction

Principal Investigator: Gilbert H. Smith, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: None

Cooperating Units: LPP, DCBD, NCI

Man Years: July 1, 1976 through September 30, 1977

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: The objective of the project is to elucidate the aspects of cell-virus interaction in mouse mammary tumor virus (MTV) infection and tumorigenesis. The approach includes the isolation and purification of intracytoplasmic A particles (putative MTV nucleoproteins cores) from primary and transplanted tumors and the purification of MMTV virions from similar sources. Analysis and comparison of the characteristics of each particle is performed in an attempt to establish the ontologic relationship between them within the framework of the replication cycle of the virus; examination and evaluation of MMTV-infected mammary gland explants by biochemical and morphological criteria during induction of differentiated function by hormone treatment (with special emphasis on the relationship of viral replication and expression of differentiated cellular functions).

Major Findings: Intracytoplasmic A particle structural proteins were analyzed for the presence of mouse mammary tumor virus antigenic determinants. Earlier observations had suggested that CAP p^{82} might be a precursor polypeptide to MMTV p^{27}; direct evidence for this was obtained by isolating CAP p^{82} from SDS-polyacrylamide gels and demonstrating by immunodiffusion that monovalent anti-MMTV p^{27} antiserum precipitated CAP p^{82} in an immunoprecipitation line showing identity with p^{27} precipitated from mouse mammary tumor virus. MMTV p^{14} and p^{10} antigenic determinants were localized on CAP p^{20-18} but were not demonstrable in CAP p^{82} suggesting that these CAP polypeptides are precursors to those MMTV non-glycosylated nucleoid proteins. Thus, CAP p^{82} either represents a precursor protein exclusively for MMTV capsid

protein p^{27} and CAP p^{20-18} exclusively a precursor for MMTV p^{14} and MMTV p^{10} or these antigenic determinants are masked within CAP p^{82} and are exposed during a cleavage event which produces p^{20-18}. Comparison of the tryptic peptide maps of these proteins is planned to distinguish between these possibilities. A manuscript describing these experiments has been submitted for publication.

The single-stranded DNA found associated with CAP was purified and characterized by hydroxapatite column chromatography and in the analytical ultracentrifuge. The sequence homology of this DNA to MMTV C DNA and to 60-70S MMTV genomic RNA was tested in collaboration with Dr. R. Michalides, Netherlands Cancer Institute. A paper describing the results of this study has been submitted for publication.

In collaboration with Dr. Barbara Vonderhaar, LPP, DCBD, C_3H virgin mouse mammary gland explants were placed in culture and exposed to various combinations of insulin, hydrocortisone and prolactin at physiological concentrations. It was found that like midpregnant mouse mammary gland, the virgin gland responded to the presence of these three hormones by cytologically differentiating and producing measurable amounts of specific milk proteins, i.e. α-lactalbumin and casein. However, unlike midpregnant mammary gland when DNA synthesis was inhibited e.g., by cytosine arabinoside then α-lactalbumin and casein synthesis was not detected but cellular differentiation proceeded as normal. The rough-surfaced endoplasmic reticulum showed distended cisternae as in the unblocked explants suggesting that protein was synthesized and transported across the endoplasmic reticulum membrane. Nevertheless milk protein synthesis could not be detected biochemically. These tissues are being evaluated immunologically for α-lactalbumin and by molecular hybridization for casein messenger RNA. A manuscript describing this project is in preparation. During this study it was noted that intracytoplasmic A particles and MMTV appeared in the epithelial cells during the induction of the rough endoplasmic reticulum but prior to synthesis of milk proteins i.e., under the influence of insulin and hydrocortisone, this phenomenon was also evident in explants where DNA synthesis was blocked.

Our earlier results demonstrating the conversion of intracytoplasmic A particle structural proteins to MMTV-like polypeptides with concomitant preservation of MMTV p^{27} antigenic determinants has been confirmed and extended in this laboratory and in others. All three internal, core-related MMTV antigenic determinants have been demonstrated to be present in intracytoplasmic A particles. This fact, coupled with the requirement of A particle morphologic integrity for proper precursor-processing has developed a strong argument that A particles represent intracellular MMTV nucleocapsid structures comprised of incompletely processed MMTV structural proteins.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3. The mouse mammary tumor virus problem has been and is being used as a relevant model for understanding human mammary neoplasia. Yet our knowledge of the intracellular events in MTV infection is essentially nil. The project is designed to provide a base to further our understanding of the intracellular events with the aim of elucidating the replicative cycle of MTV. Subsequently, it is hoped, armed

with this information, to specify the tumorigenic influence of the virus.

Proposed Course: To try to specify the tumorigenic influence of the virus.

Publications:

Longfellow, D.G. and Smith, Gilbert H.: Further Characterization of Intractyoplasmic A Particle Associated DNA. J. Virol. 17: 816-823, 1976.

Schneider, W.C. and Smith, G.H.: On the Isolation of Zymogen Granules by Zonal Ultracentrifugation and Their Nucleic Acid Content. Analyt. Biochem. (1977) in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8707-04 LMB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Relationship of Genetic and Non-Genetic Factors in Mouse Mammary Tumorigenesis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Vlahakis	Research Biologist	LMB	NCI
Other:	H.C. Chopra	Research Biologist	LTVG	NCI

COOPERATING UNITS (if any)

LTVG, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigations continue into the relationship of genetic and non-genetic factors in mouse mammary tumorigenesis. Interest is in the different lines of naturally occurring mammary tumor virus (MTV), and the genetically variable inbred mouse strains. Work is also concerned with the manner of transmission of the various MTV's, i.e. whether by female or equally well be either parent. Finally, strains of mice that develop hormone responsive mammary gland tumors and hyperplastic alveolar nodules (HAN), from which malignant mammary tumors arise are also being studied.

PHS-6040
(Rev. 10-76)

Serial No. ZO1 CB 8707-04 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: The Relationship of Genetic and Non-genetic Factors in Mouse Mammary Tumorigenesis

Previous Serial No.: ZO1 CBD 8206-03 LMBGY

Principal Investigator: George Vlahakis, Research Biologist, Laboratory of Molecular Biology, DCBD, NCI

Other Investigators: H.C. Chopra, Research Biologist, LTVG, NCI

Cooperating Units: LTVG, NCI

Man Years: July 1, 1976 through September 30, 1977

Total:	0.75
Professional:	0.5
Other:	0.25

Project Description: Objective 1, Approach 4

Objectives: To study the possibility that environmental factors may be contributing to the high incidence of spontaneous tumors in strain C3H-A^{vy}fB females.

Results: This study is complete. JNCI (58: 149-150, 1977). Tumor occurrences were similar in C3H-A^{vy}fB females in bedding consisting of low resin pine, and in females on bedding of pine plus cedar shavings. The high incidence of cancer, therefore, in C3H-A^{vy}fB could not be attributed to the routine use of cedar shavings in the bedding material.

Objectives: To compare the naturally occurring mammary tumor virus (MTV) from the high European mammary tumor strain GR, with MTV's from tumor strains C3H, A and DD.

Results: This study is complete and has been accepted for publication in JNCI. The GR-MTV proved to be less effective than C3H- and A-MTV's, but similar to DD-MTV in the production of tumors of the mammary gland.

Objectives: To determine whether or not strain DD males have the ability to transmit MTV as in the similar and possibly related strain GR.

Results: This study is complete and has been submitted for publication. There was no evidence of male transmission of MTV in strain DD when outcrossed to MTV-negative strains C57BL and BALB/c. The mode of vertical transmission of MTV in high tumor strain DD is by milk, whereas in strain GR transmission of MTV is accomplished by either parent and therefore is unique among mammary tumor mouse strains currently in use in cancer biology.

Objectives: Comparison of two milk-borne MTV's from high mammary tumor albino strains DD and RIII of European origin in MTV-negative substrain C57BL/Haag.

Results: This study is complete and is now being prepared for publication. The mammary tumor incidence and average age of tumor occurrence in C57BL/HaagfDD (with DD-MTV) and C57BL/HaagfRIII (with RIII-MTV) were similar, and therefore suggests that the two MTV's are probably related.

Collaborative studies on mammary tumors and hepatomas with other investigators both here and outside the NIH have continued again this year. Our contribution of valuable mammary tumor and hepatoma mouse strains, and our knowledge of the biology of these neoplasms make such studies desirable, and therefore are being pursued. One mammary tumor study with Dr. Y.N. Sinha of the Scripps Clinic and Research Foundation at La Jolla, California, entitled, "Evidence for The Influence of Mammary Tumor Virus on Prolactin Secretion in the Mouse," is now complete and has been submitted to Endocrinology for publication. Some time has been spent with other research workers, helping them to develop their own programs. This has consisted of discussions and of making available to them specific biological material for their own research requirements. Again this year, as has been the custom, student groups and other interested persons have been invited into the laboratory for demonstrations and discussion as part of their learning experience.

Significance to Cancer Research and the Program of the Institute: The spontaneous occurrence of mammary tumors in our mouse strains depend on the interplay of several factors: genotype, mammary tumor virus (MTV), hormones, environment. Studies of mammary tumorigenesis in mice need to be continued so that eventually mechanisms of interaction between the various factors will be understood and therefore, quite possibly, provide insight into the disease process in man.

Proposed Course: The mammary tumor experiments transferred from the Laboratory of Biology to the Laboratory of Molecular Biology are complete. One study has been published and a second accepted for publication. A third study has been submitted for publication.

Publications:

Michalides, R., Vlahakis, G. and Schlom, J.: A Biochemical Approach to the Study of the Transmission of Mouse Mammary Tumor Viruses in Mouse Strains RIII and C3H. Int. J. Cancer 18: 105-115, 1976.

Vlahakis, G.: Possible Carcinogenic Effects of Cedar Shavings in Bedding of C3H-A^{vy}fB Mice. J. Natl. Cancer Inst. 58: 149-150, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 8708-03 LMB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Modulation of Differentiated Functions in Choriocarcinoma Cells in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Stromberg	Sr. Staff Investigator	LMB	NCI
Other:	K.V. Speeg	Former Research Associate	LMB	NCI
	J.C. Azizkhan	Predoctoral Student	LMB	NCI

COOPERATING UNITS (if any)
NIAMDD, CE
NCI, LB

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Gene Regulation Section

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human choriocarcinoma is being maintained in tissue culture and the modulation of two products (chorionic gonadotropin and placental alkaline phosphatase) which are tumor markers is being examined. The ability of BCG-activated macrophages to kill human choriocarcinoma is also being examined.

PHS-6040
(Rev. 10-76)

Serial No. Z01 CB 8708-03 LMB
1. Laboratory of Molecular Biology
2. Gene Regulation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1976 through September 30, 1977

Project Title: Modulation of Differentiated Functions in Choriocarcinoma Cells in Culture

Previous Serial Number: Z01 CBD 8205-02 LMBGY

Principal Investigator: Kurt Stromberg

Other Investigators: Jane C. Azizkhan

Cooperating Units: NIAMDD, CE
NCI, LB

Man Years: July 1, 1976 through September 30, 1977

Total:	1.5
Professional:	1.0
Others:	0.5

Project Description:

Objective 1: To maintain human choriocarcinoma cells in tissue culture and to study their growth characteristics.

Findings: A) Under certain circumstances these cells will grow in serum-free media allowing experiments under defined conditions to be carried out. B) Fetal serum enhances rate of cell division, results in less HCG/mg protein and causes smaller and more numerous cells than newborn calf serum.

Objective 2: To study in detail modulation of two cell products - human chorionic gonadotropin and placental alkaline phosphatase.

Findings: A) HCG is a protein hormone induced by CAMP (or its analogs or PDE inhibitors) by a process dependent on protein synthesis and apparently independent of RNA or DNA synthesis. In fact inhibitors of both RNA and DNA synthesis result in induction of HCG.

These cells lack peak I binding protein - protein kinase (DEAE cellulose method of Gilman) under certain conditions of growth whereas normal placenta has both peak I and II. A method to induce synthesis of peak I has been found and the presence or absence of this peak is being investigated with respect to rate of HCG synthesis.

B) Alkaline phosphatase is a membrane bound glycoprotein whose function is poorly understood. It's activity can be induced with halogenated pyrimidines or butyrate. Choriocarcinoma in tissue culture contains 2 forms - one, the expected fetal isozyme which is heat stabile and shows a specific pattern of amino acid inhibition and another isozyme which resembles a liver form being heat labile and inhibited in a different pattern by several amino acids. Gene regulation is being studied by modulation of DNA and RNA synthesis.

Objective 3: To set up and test the Hibb's BCG-activated macrophage system and ascertain whether or not choriocarcinoma cells are recognized as malignant.

Finding: The Hibb's system has been set up and tested. Because normal trophoblast shows rapid growth, tissue invasion, blood supply organization and ability to metastiasize we wished to test whether the macrophage system could recognize choriocarcinoma as malignant. It could not and these cells were treated as normal. The basis for this is being explored.

Significance to Cancer Research and the Program of the Institute: Objective 1, Approach 4; Objective 3, Approach 2; Objective 4, Approach 1 and Objective 5, Approach 4.

Knowledge of the basic biology of a particular kind of human cancer is a necessary prerequisite to understanding its etiology and control. Choriocarcinoma in tissue culture represents a model system to study human malignancy. The normal counterpart of these cells (placenta) is said to possess the ability to grow rapidly, organize blood supply, invade normal tissue and metastasize. It would be highly desirable to know how this happens but this information is largely unavailble. It is difficult to study experimentally for obvious ethical reasons. Choriocarcinoma cells retain many of the normal features of placenta but have the added advantage of growing in tissue culture and being useful for experimentation. Choriocarcinoma cells possess two glycoproteins (HCG and placental alkaline phosphatase) currently being used as cancer markers with both diagnostic and prognostic value. However, those factors responsible for modulation of the markers are basically unknown but ultimately essential for the markers to be useful clinically. They are easily studied in this tissue culture system.

Moreover, the effect and mode of action of various chemotherapeutic and immunotherapeutic agents may also be assessed with these cells. It represents, therefore, a microcosm in which to conduct the war on cancer.

Proposed Course: Study of the modulation parameters alkaline phosphatase and HCG will be extended to the JAR line of choriocarcinoma cells.

Publications:

Speeg, K.V., Azizkhan, J.C., and Stromberg, K.: The stimulation by methotrexate of human chorionic gonadotropin and placental alkaline phosphatase in cultured choriocarcinoma cells. Cancer Research 36: 4570-4576, 1976.

Speeg, K.V., Azizkhan, J.C., and Stromberg, K.: Characteristics of alkaline phosphatase from two continuous lines of human choriocarcinoma cells. Experimental Cell Research 105: 199-206, 1977.

LABORATORY OF PATHOPHYSIOLOGY
ANNUAL REPORT SUMMARY
July 1, 1976 to September 30, 1977

The research effort of the laboratory is focused on the following subjects:

1. Pathophysiology of mammary tumors.
2. Regulation of cell cycle.
3. Behavior of polysomes, ribosomes, ribosomal subunits and messenger RNA related to the onset and cessation of growth in lymphocytes.
4. Structure and function of biological membranes.
5. Role of extranuclear DNA and nucleotide metabolism in normal and neoplastic tissues.
6. Pathophysiology of cachexia in the tumor-bearing host.
7. Effects of ionizing radiations on bone marrow and various tissue components.

1. Pathophysiology of mammary tissues

A process of autodigestion limited to each neoplastic cell appears to be the predominant mechanism of regression in the hormone-dependent MTW9 mammary carcinoma. The relationship between autodigestion and hormone removal is unknown. We hypothesized that hormonal deprivation alters the cytosol proteins and increases the substrate availability to the lysosomal enzymes of the cells with consequent autodigestion. In support of this hypothesis, Dr. Rouleau found that within hours after hormone removal cytosol proteins became more labile to lytic enzymes, such as trypsin, and the synthesis of 3 cytosol proteins (about 68,000, 42,000, 25,000 MW) ceased while total protein synthesis continued. Dr. Bodwin found that hormone removal was followed by a rapid disappearance of the microsomal and soluble isoenzymes of G6PD.

Dibutyryl cyclic AMP (DBcAMP) mimicked the effects of castration in producing regression of mammary carcinomas. In transplanted MTW9 as well as in the primary 7-12 DMBA-induced mammary carcinomas, DBcAMP also produced loss of soluble and particulate bound G6PD activity while estrogen injections restored growth and isoenzyme activity.

Particular attention was devoted to the specific estrogen and DBcAMP-binding proteins by Drs Cho-Chung and Bodwin. Estrogen receptors were quantitated in a variety of experimental mammary carcinomas; their translocation into the nuclei during hormone treatment was evaluated; and their reduction in tumors regressing after hormonal deprivation was measured. Cytosol of DBcAMP-responsive tumors contained cAMP-binding proteins and also cAMP-independent and -dependent forms of protein kinase with similar sedimentation constants. When tumor growth was arrested by DBcAMP, an accumulation of cAMP-binding proteins was observed in the nuclei with a concommitant increase of protein kinase activity. This nuclear transfer did not occur when the tumor was unresponsive to DBcAMP. Primary, DBMA-induced mammary carcinomas possess high estrogen-binding and low cAMP-binding proteins in the cytosol. Tumor regression induced by castration, reversed the relative proportion of the

estrogen and c-AMP-binding proteins. Growth resumption, produced by estradiol-17β, returned the relative proportion of the binding proteins to the original ratios. Interdependence between estrogen- and cAMP-binding proteins can be demonstrated during regression. Moreover, initiation of regression is characterized by the phosphorylation of a regression-associated protein, which appears to be equally dependent on estrogen or DBcAMP treatments.

The study of the rate of hormone-binding proteins in tumor growth and regression has been expanded to prolactin. Dr. Knazek utilized as an experimental model, the liver of mice in which a defect of the anterior hypophysis produced dwarfism. He showed that the liver of these mice lacked prolactin receptors; however, he was able to induce them and to study the kinetics of induction. Surprisingly growth hormone more than prolactin was able to induce detectable prolactin-binding sites; this occurred within 16 hr and cessation of treatment resulted in disappearance within 24 hr. Growth hormone induced prolactin receptors but bound to them very poorly. In studying the reasons for this apparent contradiction, Dr. Knazek observed that addition of phospholipase to cell cultures of hepatocytes prevented the loss of prolactin receptors. The role of the phospholipase and growth hormone in the maintenance and induction of the lactogenic binding sites will be investigated further.

Another aspect of mammary tumor pathophysiology is studied by Dr. Qasba and co-workers. They isolated and purified rat α-lactalbumin (αLA) and messenger RNA for αLA. Two forms of αLA have been found in rat milk, one probably a precursor of the other. Both are glycoproteins and their amino acid composition has been established. Quantitation of mRNA for αLA and casein in the lactating rat mammary gland has been obtained by translation in a cell-free wheat germ system. αLA production was found in several mammary tumors and a change in production was obtained by hormone treatment. Unexpectedly it was found that pituitary hormones increased 3-fold αLA production by DMBA mammary carcinomas but decreased to one-half αLA production by MNU-induced carcinomas. The study proceeds with the aim of testing whether quantitation of αLA and its mRNA can help to understand the interaction between growth and the functional status of secreting mammary cells, normal and neoplastic.

Following the same approach, Dr. Vonderhaar studied mammary gland development in hypothyroid mice. She observed that the gland developed only a rudimentary ductal system, however, prolactin injections in the hypothyroid host induced extensive lobuloalveolar formation. Utilizing *in vivo* and *in vitro* analysis of glands from hypothyroid animals, Dr. Vonderhaar established that: a) epithelial growth as well as αLA production is regulated by the ratio of prolactin to thyroid hormones; b) the coupling of functional differentiation with a round of cell division in the mouse mammary gland is controlled by the thyroid; and c) the epithelial cells of the gland contain binding sites for T3.

As an extension of a project initiated by Drs. Gimbrone and Brem, Dr. Maiorana is completing the evaluation of the iris transplant technique as a method to assess the angiogenic capacity of mammary tissue. The objective of this work is to develop a procedure for assessing the neoplastic potential of mammary epithelial cells that morphologically appear only hyperplastic, thus clinically "benign".

2. Regulation of cell cycle

The role of poly (ADP-Ribose) in the initition of cell division has been further studied by Drs. Kidwell, Stone and Lorimer. Poly-ADP Ribose is associated with basic chromosomal proteins, and a major objective of the group has been the characterization of this association with particular reference to cell replication. A chromosomal polymerase attaches poly(ADP-Ribose) to H1 histone. The polymer-protein complex is produced by ADP-ribosylation of H1 histone. The poly(ADP-Ribose) chain serves as a bridge linking H1 molecules to form histone dimers. The average chain length of poly(ADP-Ribose) with the complex was found to consist of 15 ADP-Ribose units. The complex is formed by two H1 molecules in association with one molecule of poly(ADP-Ribose). The complex appears to be the same in HeLa cells, human mammary carcinoma cells and normal, lactating and neoplastic mammary tissues of rats. The polymer chain length is controlled by a poly(ADP-Ribose) glycohydrolase, located in the nuclei. The enzyme has been freed of contaminating phosphodiesterases and nucleases and has been shown to degrade the polymer from the non-reducing end of the chain. It is inhibited by ADPR, cAMP, and single stranded DNA. Differences between normal and neoplastic mammary tissues in polymer degradation and its role in chromatin protein modifications are being studied.

The poly(ADP-Ribose) project has expanded in 2 directions. Dr. Stone is studying the adenylation of a small basic protein that occurs when HeLa cells are pulse-labeled with ^{3}H-adenosine and requires a component of the 105,000 g cytosol. Dr. Kidwell has developed from DMBA-induced mammary carcinoma, a cell population which show an increase of acetate incorporation upon addition of insulin and prolactin to the media. Moreover, addition of unsaturated fatty acids to the media, sharply increased cell growth; linoleic acid in particular was most effective. Analysis of rat serum showed that it contains 18-times as much linoleic acid as fetal calf serum, and rat serum was found to stimulate 2-3 times the growth of the DMBA-tumor derived cells. The role of the unsaturated fatty acids in stimulating growth of mammary epithelium will be studied further.

3. Behavior of polysomes, ribosomes, ribosomal subunits and messenger RNA related to onset and cessation of growth in lymphocytes

Human lymphocytes are used as a non-growing cell population in which an increment of protein synthesis can be induced by PHA. On the assumption that this experimental approach mimics conditions in vivo, Drs. Cooper, Berger and Harms-Ringdahl have further studied the biochemical steps controlling protein synthesis during onset and cessation of cell growth and division. In resting lymphocytes over 70% of the ribosomes are in a "free" form that does not participate in protein synthesis. After PHA treatment the increase in protein synthesis is entirely dependent on the free ribosomes, that are considered as a large reservoir ready for utilization. This reservoir is replenished at the peak of cell growth and protein synthesis as if the cell anticipates future needs. New ribosomal subunits participate in protein synthesis for 15-20 minutes and are then inactivated into free ribosomes. Inactivation occurs because of a failure of the ribosome to dissociate into 2 subunits. A dissociation factor has been shown to exist in lymphocytes as in other cells,

and the mechanism of activation is under study.

Participation of ribosomal subunits to the initiation of translation was studied using actinomycin-D as a probe. Continuous protein synthesis was required to maintain RNA synthesis. In lymphocytes protein synthesis could not be stimulated in the absence of tRNA synthesis and sharply declined immediately after tRNA synthesis was halted. There is one molecule of $tRNA_f^{met}$ for each 5 native subunit pairs and the concentration increases with PHA stimulation. Rate of protein synthesis and activation of resting lymphocytes seem to depend on $tRNA_f^{met}$ production. During lymphocyte activation, however, events have been observed to suggest that the cell prepares for cessation of growth. New ribosomal subunits appear to enter the free ribosome pool before participating in protein synthesis, possibly for lack of DF. This observation will be expanded.

The derivation of mRNA from the heterogeneous nuclear RNA (hnRNA) has been further studied by Dr. Berger who concluded that a minimum of 40% of the polyadenylated hnRNA, synthesized with exogenous label supplied during a pulse, contribute to mature, labile and stable $mRNA^+poly(A)$. In resting lymphocytes about 50% of $mRNA^+poly(A)$ was not associated with the protein synthesizing apparatus of the cell. A large amount of unbound polyadenylated mRNA disappeared. Approximately one-half of the stable mRNA was polysome-associated; the other half was divided equally between unbound material and material cosedimenting with dissociated ribosomal subunits.

The function of non-translated, very short-lived mRNA was studied in mitogen-stimulated normal lymphocytes and in the neoplastic lymphocytes of MOLT-4 populations. Labile mRNA was absent in normally growing cells but present in resting cells. In MOLT-4 cells, $mRNA^+poly(A)$ had a half-life of 2 hrs in the presence of actinomycin-D, and this decay rate was much faster than normally growing cells.

4. Structure and function of biological membranes

Dr. Pinto da Silva and his coworkers are studying structure and function of biological membranes as a way of understanding cell to cell interaction and possibly the mechanism of metastasis. Attention has been concentrated on the mechanism of cell fusion. Freeze-fracture and thin section techniques have been utilized to study the fusion of the peripheral vesicle with the plasmalemma of a phytophtora palmivora zoospore used as an experimental model. Formation and expansion of a particle-free bilayer membrane diaphragm has been demonstrated with the appearance of a polymorphic network of membrane-bound tunnels, the lumina of which are continuous with the cytoplasm. These events are also occurring in secreting cells, in particular in the milk secreting mammary gland. An analysis of the membrane residues on the surface of the fat globule, as well as the extrusion process of the globule from the cell, has revealed a surprising similarity of images with P. palmivora. It has also shown that the constitution of the membrane residues on the surface of the fat globule cannot support the belief that they are a faithful sample of the cell biological membranes. The mechanism of formation of fat globules in normal and neoplastic mammary epithelium is now being studied as an approach to evaluate the changes induced by oncogenesis.

5. Role of extranuclear RNA and nucleotide metabolism in normal and neoplastic tissues.

The project concerning identification and characterization of cytoplasmic DNA was continued by Dr. Schneider. DNA localized in mitochondrial microsomes and nuclei of hepatocytes belongs to 3 classes characterized by difference in reassociation kinetics ($C_o t_{1/2}$ = 0.03; 0.14; 500 respectively). DNA was also found in zymogen granules and its characteristics are being defined. The study of cytoplasmic DNA now proceeds in 3 directions; a) definition of band patterns for mitochondrial and microsomal DNA samples treated with restriction endonucleases, b) characterization of the DNA in cytoplasts of mammary cells by fractionation procedures and autoradiography, and c) hybridization of cytoplasmic DNA with viral DNA to search for sequences that have been already demonstrated in the nucleus. This approach aims to answer the question of whether microsomal DNA originates in the nucleus.

The modifications induced on DNA by carcinogens are being studied in the liver by Drs. Schneider and Chakrabartty, using as a probe mRNA for albumin. mRNA-alb. has been isolated from normal and trioacetamide-treated livers and has been translated in the wheat germ system. About 10% of mRNA in the liver is mRNA-alb. A 2-fold increment of mRNA-alb was produced by treatment with triacetamide. By the use of albumin cDNA probe the theory that microsomal DNA may be a "messenger" DNA will be tested. The general objective is to clarify the biological role of microsomal DNA in normal and neoplastic liver.

Drs. Anderson and Liacouras have almost completed their analysis of the role of UMP in pyrimidine metabolism. The work of the last year was mostly concerned with testing the degree of competition that cytidine and uridine analogs, such as 5-azacytidine and 5-fluorouridine, could sustain in the reactions uridine → UMP and cytidine → CMP. Results indicate that the same enzyme that phosphorylates uridine and cytidine also catalyzes the reaction with 5-azacytidine and 5-fluorouridine. Dr. Otani continues to search for amino acid derivatives with cytotoxic effects on cells. He uses the L. casei system as a screening device and concentrates on acylation as a way of reducing toxicity and chloroacetylation as a way of increasing cell penetration of the cytotoxic molecules into cells.

6. Pathophysiology of cachexia in tumor-bearing host

Dr. Morrison has reported extensively on the complex system controlling feeding and its breakdown in cachectic hypophagia. Documentation was given in support of the hypothesis that cachectic hypophagia develops as a consequence of successive deletions of different components of the feeding control system until a catastrophic collapse occurs.

Installation and testing of new equipment has been completed and a new set of experiments dealing with feeding behavior, heat production, and related changes in water uptake in relation to tumor induction and growth have been started. The extension of work in this direction was suggested by the observation that for most of tumor growth a decline in the amount of feeding activity is fully compensated by an increased feeding efficiency with no change in the final amount of food intake. This indicates that behavioral adaptation

compensates for a cachectic process which starts very early in tumor growth.

7. Effects of ionizing radiations on tissues, bone marrow in particular

Dr. Riesz and coworkers continue their work aimed at the understanding of the mechanism by which ionizing radiations produce loss of biological activity in DNA and proteins. Studies of gamma-radiolysis of deaerated neutral aqueous solutions of uracil, thymine, cytosine and the corresponding nucleosides and nucleotides showed that damage was produced by addition of H or OH radicals at the C(5) or C(6) positions. H-addition radicals to C-5 position of the 5-6 double bond of pyrimidines were made by H-abstraction from the C-6 position of the corresponding dihydro derivatives. H-abstraction from alkyl groups attached to N(1) C(6) and C(5) positions was also observed in methyl and ethyl derivatives of the dehydropyrimidines. A systematic analysis was carried on free radicals formed by the reactions of OH radicals with amides and their N-methylated derivatives, with 19 amino acids in aqueous solutions and with dipeptides in their zwitterionic forms. The effects of hydrated electrons generated by gamma hydrolysis was studied in the 20 most common amino acids in deaerated aqueous solutions and the ESR spectra from spin-trapped deamination radicals were characterized. A study of chemotherapeutic agents of the intercalating type (adriamycin, daunomycin, actinomycin-D) has been initiated in order to better understand the mechanism of potentiation of ionizing radiations produced by these substances.

Miss Uphoff used toxic doses of radiation to outline the laws governing transplantation of hybrid tissue to a parental strain. High leukemic AKR mice develop the disease in 90% of animals but lethal irradiation and bone marrow replacement keeps the animals alive. Leukemias reappear in these surviving animals several months after treatment. Leukemias of donor tissue origin occurred earlier among recipients of the most compatible marrow. Two experiments aimed at understanding the reappearance of leukemia from donor marrow have given intriguing results: a) lethally irradiated AKR mice inoculated with AKR or CBA marrow from normal young donors have a 65% incidence of leukemia. When AKR and CBA marrow are mixed and incubated for 30 min at 26°C and then inoculated into lethally irradiated AKR mice, the incidence of leukemia was only 5%. b) lethally irradiated AKR mice were inoculated with AKR marrow preincubated for 30 min at 26°C with bovine serum albumin. Incidence of leukemia was 21% if recipients were under 14 weeks of age but 78% if recipients were 17-19 weeks old. Both experimental findings are being analyzed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08224-03 LPP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Arrest of Synthesis of Proteins during Regression of Hormone-Dependent Mammary Adenocarcinoma MTW9

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Rouleau	Visiting Fellow	LPP, NCI
Other:	P. M. Gullino	Chief, Lab. of Pathophysiology	LPP, NCI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objective: To assess whether the hormone dependent regression of MTW9 mammary carcinoma can be explained by an increased lability of cytosol proteins to lytic enzymes. The work of last year demonstrated that one of the earliest events in tumor regression was an increased lability of cytosol proteins to lytic enzymes. The objective of this year's work was to establish whether a change in the protein pattern of the cytosol appeared after hormonal removal. Arrest of synthesis of 3 proteins has been observed within hours from hormonal deprivation despite continuation of total protein synthesis by the tumor at levels similar to the growing phase.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed; Double-isotope labeling of tumor proteins in vivo. Polyacrylamide gel electrophoresis in SDS.

Major Findings:

1) The pattern of incorporation of labeled leucine into cytosol proteins by the growing tumors differed from the one obtained in regressing tumors.

2) The synthesis of 3 different classes of proteins was rapidly arrested at the initiation of regression.

3) These proteins have a M.W. of about 68,42 and 25,000, respectively. Arrest of synthesis in the regressing tumor was observed as early as 6 hr after hormonal deprivation of the host.

Significance to Biomedical Research and the Program of the Institute: (NCP Objective #6, Approach #3). It aims at an understanding of the biochemical control mechanisms by which a hormone-dependent tissue regresses after hormonal deprivation. Such an understanding might improve possibilities for rational therapy in hormone-dependent human mammary tumors.

Proposed Course: Identification of the proteins which disappeared from the cytosol of the tumor within 6 hrs from hormonal deprivation of the host.

Publications:

Rouleau, M. and Gullino, P. M.: Increased Susceptibility of Cytosol Proteins to Proteolytic Digestion during Regression of a Hormone-dependent Mammary Tumor. Cancer Res. 37: 670-677, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08213-03 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Glucose-6-Phosphate Dehydrogenase Isozymes in Growing and Regressing Rat Mammary Carcinomas.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey S. Bodwin	Surgeon	LPP, NCI
OTHERS:	Yoon S. Cho-Chung	Research Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study biochemical events related to growth and regression of experimental hormonally dependent mammary carcinomas which mimic human cancers. To investigate the mechanism of microsomal G6PD activity disappearance in DBcAMP induced regressing tumors which will elucidate the role of the cyclic AMP system in tumor regression. To study the correlations between tumor regressions induced by hormonal deprivation with that induced by DBcAMP treatment. Also to study the changes in G6PD activity and enzyme pattern during tumor regression and regrowth with estrogen injection and to correlate these findings with binding activity of estrogen and cAMP in the tumor cytosol and nucleus.

PHS-6040
(Rev. 10-76)

Objectives: Previous work from this laboratory has shown that G6PD enzyme activity of rat mammary carcinomas exists in 4 enzyme forms located in mitochondrial microsomal and cytosol cell functions. Characterization of these enzymes during growth, regression and BDcAMP treatment as well as correlations of this data with estrogen and cyclic AMP binding is underway to elucidate the role of these enzymes as biological markers in the early events of tumor regression.

Methods: 1) Primary 7, 12 DMBA induced and MTW9 transplantable rat mammary carcinomas both hormone dependent were used in studies. 2) G6PD activity of tumor cytosol was assayed directly with a spectrophotometer and histochemically by staining acylamide gels after electrophoresis. 3) Partial purification of enzymes was performed with preparative disc electrophoresis.

Major Findings: 1) MTW9 carcinomas regressing after hormonal deprivation of the host show a decrease in total G6PD activity due to reduction in microsomal and soluble isozymes. 2) Treatment of MTW9 tumor bearing animals for 3 days with DBcAMP resulted in tumor growth arrest and microsomal G6PD activity disappearance. Cessation of treatment restores growth of tumor and microsomal activity. 3) Castration of 7,12 DMBA tumor bearing rats results in progressive loss of soluble and particulate bound G6PD activity while reinjection with estrogen results in tumor regrowth and progressive reappearance of soluble and particulate enzymes. 4) Glucose-6-phosphate and galactose-6-phosphate serve as substates for G6PD isozymes, but soluble and particulate isozymes demonstrate different K_m values for these substrates.

Significance to Cancer Research: (NCP Objective #6, Approach #3). These studies provide insight into the biochemical events related to growth of experimental hormonally dependent mammary carcinomas which mimic human cancers. Understanding of the mechanism of microsomal G6PD activity disappearance in DBcAMP treated regressing tumor will elucidate the role of the cyclic AMP system in the control of abnormal growth.

Proposed Course of Research:

1) G6PD isozyme activity will be used as a biochemical marker to study regression of hormone dependent and independent tumors, in conjunction with studying estrogen and cAMP binding receptors.

2) Further investigation into the biochemical and molecular events related to G6PD activity in tumor growth and regression will not be done at this time.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08223-02 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Estrogen Binding Studies in Ovarian Dependent and Independent Rat Mammary Carcinoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey S. Bodwin	Surgeon	LPP, NCI
OTHER:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study was begun to investigate the specific biochemical event of estrogen binding in relation to growth and regression of ovarian dependent and independent tumors.

Objectives: 1. To characterize estrogen binding components in cytosol and nuclei of ovarian dependent and independent tumors.

2. To investigate the functional significance of estrogen binding in relation to growth and regression of hormone dependent and independent tumors.

3. To study the relationship of estrogen receptors with other hormone receptors involved in the growth and regression of cancer cells.

PHS-6040
(Rev. 10-76)

Methods:

1. 7,12 DMBA induced primary rat carcinoma was used as experimental model of a hormone dependent tumor.
2. DMBA#1, 13762 and MTW9 A transplantable rat mammary tumors were used as hormone independent tumors.
3. Estrogen binding was measured by the dextran coated charcoal method.
4. In vivo radionuclide injection into host animals to determine cytosol and nuclear binding of cytosol.
5. Differential centrifugations and sucrose density gradient centrifugation followed standard techniques.
6. Incubation of whole tissue slices with estrogen radionuclides to determine cytosol and nuclear incorporation of estrogen.

Major Findings:

1. DMBA tumors possess specific estrogen binding in the cytosol of 30-120 fmol/mg. Whereas, hormone independent tumors 13762, DMBA#1 and MTW9A exhibit cytosol binding of 5-20fmol/mg, 1fmol/mg and 2fmol/mg respectively.
2. Nuclei of DMBA growing tumors were found to have specific estrogen receptors in the amount of 40-100 fmol/mg.
3. Scatchard analysis of binding data reveals a Kd of 10^{-10}M for DMBA cytosol, $5x10^{-10}$M for DMBA nuclei and $5x10^{-9}$M for 13762 hormone independent tumor cytosol.
4. Sucrose density gradient centrifugation shows both 8S and 4S binding components in DMBA tumor cytosol and 5S components in the tumor nuclei.
5. After hormone deprivation of host animals (ovariectomy) DMBA tumors show a decrease in both cytosol and nuclear estrogen binding, decreasing to 50% of control values 7 days after ovariectomy. Estrogen binding returns to control levels after injection of host animal with 17β-estradiol.
6. Incubation of whole tissue slices with radioactive 17β-estradiol results in nuclear accumulation in DMBA growing tumors of 90fmol/mg, 300fmol/mg in hormonally deprived, dependent DMBA tumors, and 35fmol/mg in hormonally deprived, independent DMBA (12%, autonomously growing despite ovariectomy) tumors.

Significance to Cancer Research - (NCP Objective #6, Approach #3).

By investigating the quantitative and qualitative differences in estrogen binding components of hormone dependent and independent tumors, the

functional role of estrogen receptor binding and its relation to other hormonal receptors during growth and regression of tumor cells can be elucidated.

Course of Research

1) To correlate estrogen binding capacity of tumor cytosol and nucleus with the degree of hormonal dependency of growth.

2) To investigate the functional role of nuclear binding of estradiol during growth and regression and its relation to other hormonal receptors as well as its relation to the phosphorylation of nuclear proteins.

Publications

Bodwin, J. S., and Cho-Chung, Y. S.: Dibutyryl cyclic AMP mimics ovariectomy. Glucose-6-phosphate dehydrogenase isozyme shift during hormone-dependent mammary tumor regression. Cancer Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05216-06 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Cyclic Nucleotide on In Vivo Tumor Growth Inhibition.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI
OTHER:	Timothy Clair	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: To assess whether qualitative alteration of cyclic AMP (cAMP)-binding proteins of tumors is correlated with "unresponsiveness" to dibutyryl cyclic AMP (DBcAMP)-induced tumor regression in vivo; to assess the apparent correlation between nuclear translocation of cAMP-binding proteins and DBcAMP-induced tumor regression in vivo.

PHS-6040
(Rev. 10-76)

Methods :

i) Tumors: Two cell populations isolated from Walker 256 (W256), hormone-independent mammary carcinoma; one regresses (DBcAMP-responsive) and the other continues to grow (DBcAMP-unresponsive) under DBcAMP treatment, were used.

ii) DBcAMP treatment in vivo: Dibutyryl cAMP was injected 10 mg/0.2 ml 0.85% NaCl, 1 day/200 g rat s.c.

iii) cAMP assay: Cyclic AMP levels in tumors were measured by Gilman's competitive protein binding assay.

iv) cAMP-binding assay: a) standard assay: cAMP-binding activity was measured by the competition assay of Gilman at pH 4.5 or 6.5 b) total binding assay: the competition assay of Gilman was performed at 23° for 3 hr (^{3}H cAMP-binding to proteins includes not only free sites but also endogenously bound sites by the exchange).

v) Protein kinase assay: the activity was determined by measurement of the incorporation of ^{33}P from γ-labeled ATP into histone.

vi) Cell fractionation, sucrose density gradient centrifugation and disc gel electrophoresis followed standard techniques.

Major Findings:

1) Behavior of cAMP-binding proteins and protein kinase in DBcAMP-responsive and -unresponsive W256 during DBcAMP treatment in vivo: Cytosol from DBcAMP-responsive W256 exhibits three major peaks of cAMP-binding activity, sedimenting at 4.3 S, 5.6 S, and 6.9 S, respectively. The cytosol also contains cAMP-independent and -dependent forms of protein kinase, sedimenting at 3.3 S, 5.6 S, and 6.9 S, respectively. The latter two sedimenting constants are identical to those of heavier cAMP-binding components, suggesting the relationship between cAMP-binding protein and protein kinase. In the unresponsive W256 cytosol, the binding component of 4.3 S and the protein kinase fraction of 3.3 S decreased while the higher molecular weight species of these proteins (>7S) increased. Dibutyryl cAMP treatment results in a shifting of the heavier binding and kinase components to their respective lighter components in the responsive tumor but not in the unresponsive tumor. Thus cAMP-dependent dissociation of the holoenzyme or the inactive form of protein kinase, CR, into an active, catalytic subunit, C, and a regulatory component, R (the cAMP-binding protein), is decreased in the unresponsive tumor. Moreover DBcAMP treatment (6 days) of responsive tumor resulted in a marked decrease in the cAMP-binding and protein kinase species in the cytoplasm and the concomitant increase of these protein species in the nuclei. The same treatment produces no change in the binding and kinase of unresponsive tumor cytosol or nuclei. The predominant species of cAMP-binding proteins which is increased in the nuclei of the responsive tumor has a sedimentation constant of 3.6 S. This peak also shows protein

kinase activity which is not stimulated by cAMP but is inhibited by the inhibitor protein of cAMP-dependent protein kinase. The protein substrate specificity of this increased protein kinase is similar to that of cytosol cAMP-dependent protein kinase and distinct from that of kinase present in control nuclei. Scatchard analysis of cAMP-binding data shows that the increased binding proteins in DBcAMP-stimulated nuclei of the responsive tumor exhibit two types of binding: One is identical to those present in the unstimulated nuclei, and the other is similar to one of the two types of binding fund in the cytosol. These data suggest that the nuclear accumulation of cAMP-binding proteins and protein kinase may play an important role in cAMP-mediated control of growth and that defect in this event may be correlated with "DBcAMP-unresponsiveness" in vivo.

2) Nuclear accumulation of cAMP-binding proteins and protein kinase in response to tumor slice incubation with [^{3}H]cAMP in vitro: A marked increase of cAMP-binding and protein kinase activities occurs in the nuclei of DBcAMP-responsive W256 following incubation of the tumor slices with cAMP in vitro. The macromolecular components containing [^{3}H]cAMP in the nuclei can be extracted with 1.0 M KCl and identified by polyacrylamide gel electrophoresis. Cytoplasmic origin of these increased nuclear cAMP-binding and protein kinase activities is suggested by the following observations: a) cytoplasmic and nuclear cAMP-binding and protein kinase activities are inversely related during cAMP stimulation of tumor slices; b) the sequential transfer of cAMP-binding proteins and protein kinase to the nucleus is a temperature-dependent process; c) an initial interaction of cAMP with cytoplasm is an absolute prerequisite for the nuclei binding in vitro; and d) a similarity between protein kinase of stimulated nuclei and that of cytoplasm with respect to substrate specificity and the inhibitory effect of protein kinase inhibitor protein on the enzyme. The nuclear accumulation of cAMP-binding proteins and protein kinase is greatly diminished in DBcAMP-unresponsive W256 tumor slices.

3) Nuclear binding of cAMP-binding protein-cAMP complex in a cell-free system: To examine more precisely the transfer of cytoplasmic cAMP-binding proteins into the nucleus, studies were carried out with cytosols and whole purified nuclei prepared from DBcAMP-responsive and -unresponsive W256. Cytosols, preincubated with [^{3}H]cAMP, were incubated further with nuclei, and the macromolecular-bound radioactivity recovered in the nuclear extract was identified by electrophoresis. The macromolecular-bound radioactivity peak was found in the responsive tumor nuclear extract but not in the nuclear extract of the unresponsive tumor. We next examined whether the failure of cAMP-binding proteins to bind to the nuclei of unresponsive tumor was due to defective cytoplasmic binding proteins or to a defect in the nuclear acceptor sites of these proteins. Cyclic AMP-binding protein-[^{3}H]cAMP complex derived from each responsive and unresponsive tumor cytosol was incubated with homologous or heterologous nuclei and the specifically bound-[^{3}H]cAMP in the washed nuclei was examined. The experiments showed that cytoplasmic cAMP-binding protein-cAMP complex from responsive W256 binds to isolated nuclei from both responsive and unresponsive tumors, whereas the complex from the unresponsive tumor binds neither nuclei. The

results suggest that the lack of nuclear accumulation of cAMP-binding proteins and protein kinase observed in unresponsive W256 could have been due to a defect in cytoplasmic cAMP-binding proteins which fail to interact with nuclear components. Thus a molecular lesion in cAMP-binding proteins can be a cause of DBcAMP-unresponsiveness of one cell population of Walker 256 mammary carcinoma.

Significance to Cancer Research - (NCP objective #6, Approach #3). These studies contribute to the understanding of the molecular mechanism of cAMP-induced tumor regression. Nuclear accumulation of cAMP-binding proteins and protein kinase in DBcAMP-responsive W256, following DBcAMP treatment *in vivo*, incubation of tumor slices with cAMP, *in vitro*, and in the experiments with cell-free systems, strongly suggests the important role of these proteins in cAMP-induced tumor regression. Understanding of molecular mechanism of cAMP action in growth control is essential for the clinical application of cAMP in cancer treatment.

Proposed Course of Research: To extend the investigation on the mechanism of cAMP action on growth control, the following proposal is made:

i) Purify cAMP-binding proteins and protein kinase from DBcAMP-responsive and -unresponsive W256 and characterize the physico-chemical properties of isolated proteins.

ii) Assess nuclear binding of these purified proteins in a cell-free system.

iii) Assess nuclear acceptor sites of these macromolecules.

iv) Assess the inter-relationship between cAMP-binding proteins and protein kinase in the nuclei.

v) Assess whether phosphorylation is intimately related with the nuclear binding of these proteins.

Publications

1. Cho-Chung, Y.S. and Clair, T.: Altered Cyclic AMP-binding and db Cyclic AMP-unresponsiveness in Vivo. Nature 265: 452-454, 1977.

2. Cho-Chung, Y.S., Clair, T., Yi, P.N., and Parkison, C.: Comparative Studies on Cyclic AMP Binding and Protein Kinase in Cyclic AMP-responsive and -unresponsive Walker 256 Mammary Carcinomas. J. Biol. Chem. (In press)

3. Cho-Chung, Y.S., Clair, T., and Porper, R.: Cyclic AMP-binding Proteins and Protein Kinase during Regression of Walker 256 Mammary Carcinoma. J. Biol. Chem. (In press)

4. Cho-Chung, Y.S., Clair, T., and Huffman, P.: Loss of Nuclear Cyclic AMP-binding in Cyclic AMP-unresponsive Walker 256 Mammary Carcinoma. J. Biol. Chem. (In press)

5. Cho-Chung, Y.S., and Newcomer, S.F.: Adenylate Cyclase, Cyclic AMP-phosphodiesterase and Regression of Walker 256 Mammary Carcinoma. Cancer Res. (In press)

6. Cho-Chung, Y.S.: Cyclic AMP and in Vivo Tumor Growth. In Kellen, J.A. (Ed.): Influence of Hormones in Tumor Development. Cleveland, Ohio, CRC Press, (In press)

7. Cho-Chung, Y.S.: Role of Cyclic Nucleotides on in Vivo Tumor Growth Inhibition. In Vasku, J., Erban, J., and Sule, K. (Eds.): Progress in Pathology, Proceedings from the IInd Congress on Pathological Physiology. Academia Praha (In press)

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PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Interrelationship of Cyclic Nucleotide and Steroid Hormone in Growth Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoon Sang Cho-Chung	Research Chemist	LPP, NCI
OTHERS:	Jeffrey S. Bodwin	Surgeon	LPP, NCI
	Timothy Clair	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objective: To assess the intervention of cyclic AMP in the action of estrogen in the growth and regression of hormone-dependent mammary tumors. To assess whether tumor regression induced by either ovariectomy or dibutyryl cAMP treatment occurs through a similar biochemical mechanism.

PHS-6040
(Rev. 10-76)

Method:

i) Tumors: Primary 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinoma; regression is obtained by either bilateral ovariectomy or dibutyryl cyclic AMP (DBcAMP) treatment of host animal (10 mg/0.2 ml, 0.85% NaCl/200 g rat/day s.c.).

ii) estrogen binding assay: dextran coated charcoal method

iii) cAMP assay: cyclic AMP levels in tumors were measured by Gilman's competitive protein binding assay.

iv) cAMP-binding assay: the competition assay of Gilman was performed at 23° for 3 hr.

v) Protein kinase assay: the activity was measured by the determination of the incorporation of ^{33}P from γ-labeled ATP into histone.

vi) Adenylate cyclase assay: by the method of Krishna et al. ± NaF utilizing AMP-PNP (ATP analogue) as the substrate.

vii) cAMP-phosphodiesterase assay: used method of Thompson and Appleman.

viii) Cell fractionation, sucrose density gradient centrifugation, and disc gel electrophoresis followed standard techniques.

Major Findings:

1) DBcAMP treatment mimics ovariectomy in producing the inverse-relationship between cAMP-and estrogen-binding activities in regressing DMBA tumors: Growth of DMBA mammary tumors is arrested by either ovariectomy or DBcAMP treatment. When this occurs, a change in the specific binding of estrogen and cAMP to the proteins in the cytosol and nuclei of tumors is observed. Growing tumors possess a high estrogen-binding and low cAMP-binding in their cytosol and nuclei; whereas in the cytosol and nuclei from regressing tumors, cAMP-binding was increased 3 and 5 fold, respectively, and estrogen-binding was decreased by 70% and 50%, respectively. These changes in estrogen- and cAMP-binding activities began within one day after ovariectomy or DBcAMP treatment and the changes were reversed when resumption of tumor growth was induced by either the injection of 17β-estradiol or cessation of DBcAMP treatment. When DMBA tumors (~12%) failed to regress after ovariectomy or DBcAMP treatment, the change in estrogen and cAMP-binding activities does not occur. Significant increases in the cAMP level as well as in adenylate cyclase and cAMP-phosphodiesterase activities are also found in the regressing tumors. Concomitant with the increase of cAMP-binding activity is an increase in histone kinase activity in the regressing tumor. The data suggest an inter-relationship between the steroid and cAMP in the growth control of a hormone-dependent mammary tumor.

2) A new non-histone protein phosphorylation in the nuclei of regressing

DMBA tumors following ovariectomy or DBcAMP treatment: Nuclei from growing and regressing (post ovariectomy or DBcAMP treatment) tumors were incubated with [γ-^{33}P] ATP, and the phosphorylated nuclear proteins were separated on SDS-acrylamide gel electrophoresis. In growing tumors, a low molecular weight basic protein species was the major endogenous substrate for nuclei-bound protein kinase; whereas, in nuclei from regressing tumors, a new non-histone protein species became the predominant substrate of the endogenous kinase. Phosphorylation of this regression-associated-protein (RAP) began within one day after ovariectomy or DBcAMP treatment and stopped when resumption of tumor growth was induced by either the injection of 17β-estradiol or cessation of DBcAMP treatment. When DMBA tumors failed to regress after ovariectomy or DBcAMP treatment, phosphorylation of RAP did not occur.

The phosphorylation of nuclear proteins measured in this study is dependent on the activity of protein kinase present in the nucleus. The activity of protein kinase in the nuclei of growing and regressing tumors was, therefore, measured utilizing exogenous substrates of histones and casein. Protein kinase from growing tumor nuclei exhibited an activity of casein phosphorylation 3-fold higher than that of histone phosphorylation. DBcAMP treatment or ovariectomy resulted in a change in the protein substrate specificity of nuclear protein kinase. In the regressing tumor nuclei, the histone kinase activity increased 3-fold while the casein kinase activity decreased to 30% of that in the growing tumor nuclei. Cyclic AMP had no effect on the activity of kinase from either growing or regressing tumor nuclei, whereas, protein kinase inhibitor protein, inhibited the kinase from growing and regressing tumor nuclei, 10% and 80%, respectively. These results suggest that the differential phosphorylation of nuclear proteins in growing and regressing tumors, is probably due to the presence of different protein kinases in the respective tumor nuclei. This study presented the first evidence of a differential endogenous phosphorylation of nuclear proteins during growth and regression of hormone dependent mammary carcinoma.

Significance to Cancer Research - (NCP objective #6, Approach #3):

These studies contribute to the understanding of mechanism of hormone-dependent growth. The study shows the first evidence that action of estrogen and cAMP may be reciprocally related in the growth control of hormone-dependent mammary tumors, thus would contribute to the better treatment and prevention of malignant hormone-dependent growth.

Proposed Course of Research:

i) Assess the inter-relationship between cAMP and estrogen in the nuclear-binding in a cell-free-system.

ii) Assess the nuclear acceptor sites for cAMP- and estrogen binding proteins.

iii) Assess whether a modification of chromatin proteins or DNA would influence the nuclear-binding of cAMP and estrogen.

iv) Assess whether nuclear phosphorylation is interrelated with the nuclear-binding of estrogen and cAMP.

v) Identify regression-associated-protein (RAP).

Publications

1. Cho-Chung, Y.S., and Redler, B.H.: Dibutyryl cyclic AMP mimics ovariectomy. Growth arrest and phosphorylation of a non-histone nuclear protein in hormone-dependent mammary tumor. Science (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05219-06 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
In Vitro Simulation of Hormone-dependent Mammary Tumor Regression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Knazek	Senior Investigator	LPP, NCI
OTHER:	P. M. Gullino	Chief	LPP, NCI
	S. Chen Liu	Chemist	LPP, NCI
	M. Lippman	Senior Investigator	Med. Br., NCI
	H. Chopra	Senior Investigator	Viral Oncol., NCI
	P. Bungay	Senior Investigator	BEIB, DRS

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathophysiolgy
SECTION
Cell Cycle Regulation Section
INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A bundle of tube-shaped semi-permeable membranes, when perfused with tissue culture medium simulates the in vivo capillary bed. Isolated cells injected into the extracapillary space receive nutrients and have cell products removed by diffusion through the capillary walls. Within this artificial capillary network the environment is nearly physiologic permitting the cells to form solid masses of tumor. The effects of hormones on these target tissues are being studied both in vitro and in vivo to determine the mechanisms of hormone-dependent growth and regression. Specific hormone receptors are being studied and isolated to complement these studies. The liver of the Snell dwarf mouse has been shown to lack prolactin binding sites. These receptors were, however, rapidly induced by treatment with growth hormone and disappeared upon cessation of therapy. Studies, however showed that growth hormone alone was insufficient, rather requiring phospholipase to maintain the activity of the binding sites in vitro. This indicates that cellular phospholipids are involved in the specific binding of prolactin to cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To reproduce and study in vitro, the hormone-dependent regression process of mammary tumors observed in vivo.

Methods Employed: Established cell lines placed within artificial capillary beds form solid tumor masses in vitro. The capillary network, perfused by tissue culture medium, maintains the stability of the pericellular microenvironment while providing nutrient support and waste product removal for the cells. This technique provides the most physiologic conditions yet obtainable for cell growth in vitro.

JEG-7 is an established, cloned line of human choriocarcinoma cells.

An assay for prolactin and growth hormone receptors has been developed using a modification of the Shiu-Friesen method to determine the ability of cell membranes to bind polypeptide hormones. In addition, affinity chromatographic technique have been adapted to purify these receptors from other membrane components by dissolving membranes in detergent, then adsorbing and subsequently eluting them from a growth hormone affinity chromatography column.

Snell dwarf mice are autosomal recessive mutants whose circulating levels of growth hormone and prolactin are much lower than their phenotypically normal littermates. These animals, consequently, attain only 1/4 of the normal weight.

Major Findings: JEG-7 cells attained tissue-like density within a artificial capillary culture unit. A needle-like platinum oxygen electrode, when inserted into the extracapillary space, showed the PO_2~ 150 mm Hg. When the perfusion was halted, the pO_2 fell to near zero within 3 minutes, indicating that the metabolic requirements of such a large tissue mass were high and that a rapid and continuous perfusion was needed to supply oxygen in sufficient quantity. In-line, membrane-type oxygen electrodes and the necessary electronic support circuits have been built to measure oxygen consumption by these solid in vitro tumors and are currently being tested.

Prolactin (PRL) binding sites were shown to be absent in the livers of Snell dwarf mice (dw/dw). Phenotypically normal littermates (DW/?), however, bound PRL with an apparent affinity constant = 15 x $10^9 M^{-1}$ and possessed quantities of binding sites equivalent to 25 and 8 fmoles per mg liver protein in females and males, respectively. The binding sites were induced in livers of dw/dw mice upon transplantation of intact DW/? pituitaries under the kidney capsule, induction occurring in a biphasic manner. Detectable binding sites could not be reinduced by daily single PRL injections into dw/dw after the transplanted pituitary had been excised. Intraperitoneal injections of GH + PRL + thyroxine every 4 or 8 hours induced detectable binding sites that were maximally detectable 8-12 hours after the final injection. Injection of GH alone every 8 hours also induced detectable prolactin binding sites as early as 16 hours after the first injection, reaching maximal levels of 50 fmoles/mg protein after 32 hours of treatment. Cessation of GH therapy resulted in a disappearance of active PRL binding sites within 2 days. Single daily injections were ineffective in inducing receptor activity.

DW/? males exhibited a 6.5-fold or 2-fold increase in the number of lactogenic binding sites when treated every 4 hours with GH or PRL, respectively. Addition of PRL + thyroxine to the GH injections lowered the increase to 2-fold. ^{125}I-bGH did not bind to either the dw/dw or the DW/? membrane preparations. GH competed with ^{125}I-PRL for the receptor induced in the dw/dw only when extremely high GH concentrations were used, probably due to PRL contaminating the GH preparation in small amounts. Injection of PRL in amounts approximating the contamination in the GH preparation, did not induce the PRL receptor. This indicates that GH is the hormone responsible for the induction of lactogenic binding sites.

The ability of GH to induce the receptor activity in spite of its inability to bind to the membrane preparation suggests either an intermediate GH-responsive tissue that controls receptor induction or the presence of GH binding sites within the liver that are undetectable by receptor assays currently in use.

Isolated DW/? hepatocyte suspensions were prepared by infusing collagenase into the portal vein and then dispersing the tissue. The washed cell suspensions incubated in tissue culture medium at 37^{o}C were observed to lose $\sim$ 50% of their ability to bind PRL within a half hour, and $\sim$ 100% within 24 hours, in spite of the presence of GH in the medium. Addition of 0.1 µg phospholipase A_2 (PLA_2) per ml prevented this loss for the first 1/2 hour. Furthermore, if the cells were incubated with PLA_2 at 0^{o}C for 1/2 hour prior to assay, the specific binding of PRL then increased 30-50%. The apparent affinity constant of the receptor is approximately 0.3 x $10^{9}M^{-1}$ and is unchanged by exposure to PLA_2. Thus, removal of the β position fatty acid from cellular phospholipids appears to enhance the binding of prolactin to the cell.

Livers excised from the female rats were pulverized in liquid nitrogen, homogenized, and dissolved in Triton-X. The 100,000 x g supernatant of this suspension contained the solubilized hGH receptor as demonstrated by specific binding to ^{125}I-labeled hGH. After this receptor solution was adsorbed to an hGH-affinity column, the purified liver hGH-receptor was eluted by 5 M $MgCl_2$ and susequently dialyzed to remove the $MgCl_2$. The fractions containing the highest binding activity were injected into rabbits to develop an antibody to the hGH receptor. Subsequent assays of the rabbit sera indicated that anti-rat hGH-receptor antibodies had not been generated.

Significance for Cancer Research: An instrument that measures oxygen consumption by cells growing within the artificial capillary network may provide a sensitive and rapid means by which both early and long term effects of hormones and chemotherapeutic agents may be studied on solid tumors in vitro.

Demonstration that prolactin receptors are induced by exogenous somatotrophic hormones indicates that growth hormone may play the central role in the responsiveness of mammary carcinoma to hormonal stimuli.

Prolactin receptors have not been maintained in any of the existing cell lines. Modification of the membrane phospholipid structures by, for example,

phospholipase, may permit maintenance of receptor activity, allowing study of these regulatory structures in vitro.

Furthermore, association of phospholipids with the prolactin receptor may help to explain the correlation of certain dietary fats with the incidence of mammary carcinoma in various countries throughout the world.

Plan: Cell lines of mammary carcinoma will be cultured within the artificial capillary units and then exposed to steriod and pituitary hormones. The oxygen utilization will be followed to determine if this is a valid parameter to detect early responses to hormonal changes.

An assay will be established to determine phospholipase activity in both the tumors and livers of animals that have been hormonally stimulated or deprived.

C3H mice will be maintained on unsaturated fatty acid-free diets to determine the role of essential fatty acids on the maintenance of the prolactin receptor in the liver.

The prolactin receptor will be isolated from mouse liver to study the role of its phospholipid moiety upon binding activity.

Publications:

Knazek, R. A., Lippman, M. E., Chopra, H. C.: Formation of Solid Human Mammary Carcinoma in vitro. J. Natl. Cancer Inst. 58: 419-422, 1977.

Lippman, M., Osborne, C. K., Knazek, R. A., and Young, N.: In vitro Model Systems for the Study of Hormone-dependent Human Breast Cancer. New England J. Med. 296:154-159, 1977.

Knazek, R. A., S. C. Liu, and P. M. Gullino: Induction of Lactogenic Binding Sites in the Liver of the Snell Dwarf Mouse. Endocrinology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08205-06 LPP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Application of the Artificial Capillary Culture Technique to Polypeptide Hormone Production.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.A. Knazek	Senior Investigator	LPP, NCI
OTHERS:	P.M. Gullino	Chief, Lab of Pathophysiology	LPP, NCI
	J. Roth	Chief, Diabetes Branch	NIAMDD
	M. Lesniak	Chemist, Diabetes Branch	NIAMDD

COOPERATING UNITS (if any)

Diabetes Branch, NIAMDD

LAB/BRANCH

Laboratory of Pathophysiology

St

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The artificial capillary culture technique provides a pericellular microenvironment that closely resembles the in vivo state. This may allow normal cells to maintain their differentiated functions for prolonged periods of time in vitro. Cells are mainteined in a physiologic state without many of the artifactual conditions imposed by more standard methods of tissue culture. The technique is therefore, especially well suited for the culture of cells of endocrine origin. Response of the cells to various physiologic agents are being studied as well as the secretion of various hormones over long periods with the possible application to continuous production of hormones for laboratory and clinical uses.

Attempts to establish human cell lines that secrete significant quantities of growth hormone or insulin have been unsuccessful. While several cultures of a human pituitary adenoma have continued to secrete 2 µg prolactin per day for a period in excess of 6 months, there is neither evidence of increasing prolactin nor indications that these cells have become established.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Establishment of a human pituitary cell line that produces prolactin or growth hormone. Development of a method that provides both large quantities of polypeptide hormones and a means for studying their synthesis and secretion.

Methods Employed: Human pituitary and pancreatic tumors were enzymatically dispersed into single cell suspensions and either placed in monolayer culture or in the extra-capillary space of an artificial capillary culture unit.

Modification of the artificial capillary perfusion circuit into a single pass mode of operation enables cells inoculated into the extracapillary space to be pulsed with pharmacologic or physiologic agents. The influence of these substances on the kinetics of secretion may then be studied under conditions that approach those of the *in vivo* state.

Sera were obtained from acromegalic patients and normal volunteers and the IgG from these samples purified on DEAE cellulose columns. Aliquots of either IgG concentrates or SV-40 were added to replicate monolayer cultures of enzymatically dispersed human pituitary tumor cells. Spent tissue culture medium was periodically replaced with fresh medium and assayed for hGH of hPRL by means of specific radioimmunoassays.

Major Findings: As the project has previously shown, cells obtained from enzymatically dispersed acromegalic pituitary adenomata will continue to secrete hGH for approximately 4-6 weeks in both monolayer and capillary cultures. This inability to maintain high levels of secretion would seem to be an *in vitro* deficiency of some substance, as yet unknown, that is present in human sera. The possibility exists that acomegalic patients may contain high levels of such a factor similar to LATS, an IgG present in some patients with Graves disease.

Aliquots of acromegalic or normal IgG concentrates were added to replicate monolayer cultures of a dispersed acromegalic pituitary adenoma. One half of the medium in each culture tube was replaced every other day with fresh medium with the appropriate type and amount of IgG. One series of cultures were exposed to SV-40 on the eighteenth day of culture. The cultures have been maintained for more than 150 days. Initial values of hGH secretion were in excess of 200 ng/day but gradually decreased to less than 10 ng/day. The rates of hGH secretion appeared to be unresponsive to the additions of either IgG or SV-40. Rates of hPRL secretion initially ranged from 4000 to 7000 ng/day but decreased gradually to 2000 ng/day, being similarly unaffected by supplements of IgG or SV-40.

A human insulinoma was enzymatically dispersed and inoculated into a capillary culture unit. The circuit was maintained in its recirculating mode of perfusion for 7 days prior to alteration into the single pass mode of operation. The feed medium was then changed to one containing 200 µg

tolbutamide per ml. Within 3.5 minutes the rate of insulin secretion increased from the basal level of 0.9 ng per minute to 3.3 per minute. The elevated secretory rate persisted for 15 minutes but gradually fell to basal levels despite the continued presence of the drug in the perfusate. This observation virtually duplicates that seen when the pancreas is isolated and perfused ex vivo with tolbutamide.

Significance for Cancer Research: The characteristics of the capillary culture unit enable rapid responses to both stimuli and inhibitors of cell function to be studied in a nearly physiologic in vitro environment.

Secretion of hGH by in vitro cultures of pituitary cells has not been maintained at significant levels for more than 6 weeks. However, hPRL secretion has been maintained at high rates for long periods, reconfirming earlier observations that in vitro culture techniques may be useful in producing significant quantities of hPRL for use in studies of hormone dependent breast cancers.

Proposed Course of Research: Pituitary tumors will continue to be cultured in vitro in an attempt to establish hPRL- or hGH-secreting cell lines.

Publications:

Knazek, R. A. and Skyler, J. S.: Secretion of Human Prolactin in vitro. Proc. Soc. Exp. Biol. Med. 151:561-564, 1976.

Knazek, R. A. and Skyer, J. S.: Production of Polypeptide Hormones in Tissue Culture. Proc. of Third Int. Symposium on Growth Hormone and Related Peptides, 1975. Excerpta Medica, The Netherlands p386-395, 1976.

Skyler, J. S., Lovenberg, W., Rogol, A. D. and Knazek, R. A.: Characerization of Growth Hormone and Prolactin Produced by Human Tumors in Culture. Endocrinology 100: 283-291, 1977.

Knazek, R. A.: Endocrine Cell Culture on Artificial Capillaries. Pancreatic Beta Cell Culture, Fifth Workshop Conference Hoechst, Excerpta Medica, Amsterdam, The Netherlands (in press)

Knazek, R. A.: Tissue Culture Using the Artificial Capillary System. AIChE Symposium Series (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08230-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hormonal Control of Mammalian Follicular Maturation and Oogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. G. Hillier	Visiting Fellow	RRB, NICHD
OTHER:	G. T. Ross, M.D.	Associate Clinical Director	CC
	R. A. Knazek	Senior Investigator	LPP, NCI
	A. J. Zeleznik	Visiting Fellow	Diabetes, NIAMDD
	A. C. Rabson	Scientific Director, DCBD	LP, NCI

COOPERATING UNITS (if any)
Laboratory of Pathology, NCI
Reproduction Research Branch, NICHD
Diabetes Branch, NIAMDD

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of intraovarian sex hormone in mediating gonadotrophic stimulation of follicular maturation, including atresia and oogenesis is being studied in vivo and in vitro. These studies indicate that estrogens stimulate while androgens inhibit granulosa cell proliferation and enhance atresia during pre-antral stages of follicular maturation. Since ovarian biosynthesis of androgen requires LH stimulation, but biosynthesis of estradiol requires FSH stimulation, the role of gonadotropins in stimulating both growth and atresia is mediated by sex steroid hormone synthesis.

PHS-6040
(Rev. 10-76)

Objectives: These studies are directed toward understanding the role of gonadotropins and sex steroid hormones in initiating and regulating follicular maturation leading to atresia and ovulation in mammalian ovaries. The observation that endogenous androgens stimulate atresia in ovaries of estrogen-primed hypophysectomized immature female rats has been pursued.

Findings: Previous in vivo studies were consistent with a regulatory role for androgen during follicular maturation. Following the demonstration of a specific cytoplasmic androgen receptor with evidence for nuclear translocation of testosterone in granulosa cells from estrogen-primed hypophysectomized immature rats (HIFR), the biochemistry of androgen action in these cells has been examined further. A technique has been established for the isolation and culture of granulosa cells from estrogen-primed HIFR. Progesterone production by primary granulosa cells was shown to be stimumated by testosterone in a dose- and time-dependent fashion. This response was modulated in the presence of estrogen. Follicle stimulating hormone (FSH) but not human chorionic gonadotropin (hCG) also stimulated progesterone production by the cultured cells, moreover, further substantial increases in progesterone production were observed when cells were coincubated with FSH plus testosterone. The augmentation of progesterone production by testosterone was suppressed when steroidal or non-steroidal antiandrogens were included in the culture medium, suggesting that this effect of androgen was mediated via an interaction with androgen-specific receptors. These studies have provided further evidence for a direct role of androgen in the control of follicular maturation.

Granulosa cell differentiation occurring during preovulatory follicle maturation is characterized by an increase in the number of available cell membrane luteinizing hormone LH/hCG receptors and by intracellular modification leading to enhanced capacity to secrete progesterone. Studies were initiated to determine if these biochemical markers of granulosa cell maturation are intrinsically related. Granulosa cells have been isolated from the ovaries of estrogen-primed HIFR receiving graded doses of FSH in vivo and their ability to bind radioiodinated hCG compared at the time of isolation and following culture in vitro in the presence of various trophic agents. The accumulation of immunoreactive progesterone in culture medium has been monitored simultaneously and used as an index of steroidogenic capacity. Results of these experiments indicate that the initiation of steroidogenesis by FSH in the developing granulosa cell is antecedent to LH/hCG receptor induction. They further show that steroidogenic capacity and the induction of LH/hCG receptors are independent phenomena, suggesting that each may be under separate regulation.

Plan: In order to further extend these studies' a fully functional granulosa cell line which would be continually available for more elaborate in vitro studies is required. Currently attempts are being made to establish such a cell line using a combination of viral transformation and cell fusion techniques. Two granulosa cell lines have been established by SV-40 transformation but have neither steroidogenic capacity nor the ability to bind estrogen, progesterone, LH, or FSH. Both cell lines, however, do form tumors in nude mice.

Significance to Cancer Research: An intraovarian site of action of sex steroid hormone control of ovulation constitutes another locus with potential for voluntary reversible control of mammalian ovulation. The development of a granulosa cell line may permit study of ovarian tumors *in vivo* and *in vitro*.

Publications:

Hillier, S. G., Knazek, R. A., and Ross, G. T.: Androgenic Stimulation of Progesterone Production by Granulosa Cells from Preantral Ovarian Follicles: Further *in vitro* Studies using Replicate Cell Cultures. Endocrinology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08218-02

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of rat α-Lactalbumin.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pradman K. Qasba	Visiting Scientist	LPP, NCI
OTHER:	Pran Chakrabartty	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

NIH, NCI, Bethesda, Maryland 20014

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two molecular forms of α-lactalbumin with apparent molecular weights of 21,800 (α-LA_L) and 22,500 daltons (α-LA_H) were separated from rat milk. The α-Lactalbumins were purified to homogeneity by gel filtration with Sephadex G-150, followed by DEAE-cellulose chromatography. Both α-lactalbumins are glycoproteins. αLA_L has one residue of galactose and one residue of hexosamine more than α-LA_H. α-LA_H has one lysine, one glutamic acid and four proline residues more than α-LA_L. The two α-LA species can be descriminated by rat or cow galactosyltransferase in a lactose synthase assay, but are antigenically similar in a radioimmunoassay.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To purify and characterize rat α-lactalbumin, and thus be able to study the regulation of the synthesis of this differentiated function in normal and neoplastic mammary tissue.

Methods: α-Lactalbumin was separated from whey proteins on Sephadex G-150 columns and then further purified on DEAE-cellulose column. Purification was monitored by enzymatic methods using cow galactosyl transferase as a protein in a lactose synthetase assay.

Major Findings: 1) Two species of rat-α-lactalbumin are separable on DEAE-cellulose columns. 2) Both α-lactalbumin are glycoproteins. This finding has been unusual as the α-lactalbumin from other species are not glycosylated. 3) The molecular weight of the two α-lactalbumins αLA_L and αLA_H, is 21,800 and 22,500 daltons on SDS gels. The molecular weight of αLA_L species was also determined by equilibrium sedimentation analysis which gave a value of 21,500 ± 600 daltons. Molecular weight of α-lactalbumin from other species like, human and cow has been shown to be 16,000 daltons. This makes the rat α-lactalbumin unique in that it is a glycoprotein and is about 186 amino acid long. In contrast cow and human α-lactalbumin have 123 amino acid residues and are not glycoproteins. 4)The two species of rat α-LA differ slightly in carbohydrate and amino acid composition, (i) αLA_L has 1 mole of galactose, and 1 mole of hexosamine more than αLA_H (ii) αLA_H has 4 proline, 1 lysine and 1 leucine residue more than αLA_L. 5) The two αLA-species can be discriminated by rat or cow galactosyl transferase in a lactose synthase assay. 6) The two αa-LA species are indistinguishable by radioimmunoassay.

Proposed Course of Research: 1) N-terminal amino acid sequence of the two α-LA will be carried out in collaboration with Dr. Rae Greenberg from U. S. Department of Agriculture, USDA-ERRe, Philadelphia.

Significance to Cancer: Characterization of α-Lactalbumin will help us in understanding the mechanism involved in the synthesis of this differentiated unction in normal and neoplastic tissue.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08219-02 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Isolation and Purification of Rat α-Lactalbumin and Casein m-RNA and their cDNA Probes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pradman K. Qasba	Visiting Scientist	LPP, NCI
OTHER:	Pran Chakrabartty	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two species of α-lactalbumin (αLA) are separable from rat milk, both of which are active in a lactose synthetase assay. The two α-LA's differ in apparent molecular weights (22,500 and 21,800 daltons respectively) and net negative charge. Enzymatically and by radioimmunoassay, α-LA represents about 6% of the total protein of rat milk and 0.6% of the 3-5 day lactating rat mammary gland protein. We have quantitaed the mRNA for α-LA and casein in the lactating rat mammary gland by translating the mRNA in a wheat germ system. The in vitro synthesized α-LA and casein were identified on SDS-polyacrylamide gels after immunoprecipitation with rat α-LA or casein antibody. Thirty percent of the total protein synthesized in vitro from total cellular RNA was precipitated with α-LA antibody, and the remaining 70% with casein antibody. Proteins synthesized in vitro with poly A-bearing RNA isolated from total cellular RNA by either dT-cellulose or Poly(U)-sepharose columns, consisted of 40-50% α-LA with casein as the remainder. After fractionation of poly A RNA on sucrose gradients, α-LA message sediments as an 8.2 S peak and represents nearly 50% of the total active message.

Project Description:

Objectives: To purify and characrerize rat α-lactalbumin and casein mRNA and their complementary DNA probes so that one is able to study the regulation of the synthesis of these messenger RNA's; a) during development of mammary gland; b) in neoplastic mammary tissue; and c) the influence of hormones on their synthesis.

Methods: 1) Isolation of mRNA on Poly-U-sepahrose or dT cellulose columns and further purification on sucrose gradients. 2) In vitro protein synthesizing system derived from wheat germ which is dependent on exogeneous mRNA. The synthesis of specific proteins is measured by immunoprecipitation method.

Major Findings : 1) α-LA represents about 6% and casein about 70-80% of the total protein in rat milk. α-LA comprises about 0.6% and casein about 8-10% of the 3-5 day lactating rat mammary gland protein. 2) α-LA and casein mRNA from the lactating mammary gland was quantitated by translating the mRNA in a wheat germ system. α-LA and casein synthesized in vitro electrophoresed on SDS-polyacrylamide gels with α-LA and casein markers respectively. 3) From total cellular RNA, α-LA mRNA represents 30% of the total mRNA activity with casein mRNA representing the remaining (70%) activity. 4) In Poly A-bearing RNA, isolated from total cellular RNA, α-LA represents 40-50% of the total poly A-bearing mRNA activity, and casein represents the reamining 50-60%. 5) After fractionation of poly A RNA on sucrose gradients, α-LA message sediments as an 8.2 S peak and represents nearly 50% of the total active message. 6) α-LA mRNA activity comprises at least 90% of the total activity in a purified preparation. 7) α-LA mRNA on urea-agarose gel electrophoresis gave a single band of 8.8 S value. Casein mRNA under identical conditions gave two bands one being 12S and the other 14S.

Further Course of Research: 1) Synthesis and characterization of c-DNA probes for these mRNA's.

Significance to Cancer: DNA probes for a-LA and casein will give a very sensitive method of detecting the levels of mRNA in mammary neoplastic tissue.

Publications:

Chakrabartty, P. K., Qasba, P. K.: Partial Purification of Rat α-Lactalbumin m-RNA. Nucleic Acids Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08217-03 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Synthesis of Mammary Specific Proteins - Casein and α-Lactalbumin - in Mammary Carcinoma Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Pradman K. Qasba Visiting Scientist LPP, NCI

OTHER: P. M. Gullino, Chief, Laboratory of Pathophysiology, NCI

COOPERATING UNITS (if any)
None

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0.5	0.25	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two species of α-Lactalbumin (αLA) have been found in rat milk and both are active in a lactose synthetase assay. The two αLA's differ in apparent molecular weights (22,500 and 21,800 daltons respectively) and net negative charge. A radioimmunoassay (RIA) was developed and used to detect αLA in rat mammary carcinomas. In this assay unlabeled antigen competed against ^{125}I labeled antigen for binding to antibody against pure rat αLA of low molecular weight. However, the two α-lactalbumins are indistinguishable by the assay which is able to detect 3-5 nanograms of αLA. Primary carcinomas induced in rats by 7-12 Dimethylbenzanthracene (DMBA) or methylnitrosourea (MNU) contained αLA in quantities equal to or less than 10% the amounts found in 3-5 day rat lactating mammary gland. αLA was found only in R3230 AC and MTW9 but not in DMBA #1, Ascites 13762 and MTW9A transplantable mammary carcinomas. Transplantation of a pituitary gland under the kidney capsule of the host (a) increased αLA content of primary DMBA induced mammary carcinomas, (b) reduced αLA content of primary MNU induced mammary carcinomas and (c) was unable to modify the αLA levels in 5 transplantable mammary carcinomas.

Project Description:

Objectives: To study the production of α-lactalbumin and casein in mammary carcinoma cells and the influence of hormones on their synthesis in the neoplastic tissue.

Methods: Two species of α-lactalbumin-αLA_L and αLA_H are separable from milk. Antibody against αLA_L was raised in rabbits. Levels of αLA were measured by a radioimmunoassay using a double antibody precipitation method. Rat casein was purified on DEAE-cellulose column . Three molecular species with a molecular weight of 42,000, 29,000 and 25,000 daltons were separated. Antibodies were raised against the mixture of the three casein. The levels of casein were measured by a competitive radioimmunoassay.

The quantity of casein or αLA in the unknown sample was determined by competitive inhibition assay, i.e. casein or αLA in the sample competed with the binding of the ^{125}I-labeled casein or αLA, thus quantification was based on the reduction of precipitated counts.

Major Findings: αLA was present in all primary tumors arising from the parenchyma of virgin glands and induced by 2 different carcinogens - 7-12-Dimethylbenzanthracene and Methylnitrosourea. The average tissue level, however was only about 10% that of a 5-day lactating gland. When attempts were made to modify these levels, rather unexpected results were observed. Pituitary transplants, as well as continuous hormonal stimulation during transplantation, were unable to modify the αLA content of 4 transplantable tumors - DMBA #1, R3230AC, MTW9A, 13726MAT cells. Contrary, pituitary transplants in hosts bearing primary mammary carcinomas did induce a change in the αLA content of the neoplastic tissues. In DMBA-induced tumors, αLA was increased, by about 3-fold, but in MNU-induced tumors it was decreased by about 50% despite identical experimental conditions. We observed lack of any increasement of αLA expression in DMBA #1 tumors, when transplanted for 146 generations in lactating females, where the feasibility of cell selection was favored by an appropriate hormonal milieu maintained over a long period of time.

Proposed Course of Research: 1) Measure levels of casein and the influence of pituitary transplant on its levels in various tumors. 2) Analysis of the conditions which modify αLA and casein production in primary and transplantable tumors.

Significance to Cancer: Understanding the response of several mammary tumors to varius hormonal manipulations will help in designing experiments for early detection and appropriate treatment of breast cancer.

Publications:

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08231-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Synthesis of α-LA and Casein mRNA, during Gestation and Lactation of Rat Mammary Gland, and in Rat Mammary Carcinomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hira Lal Nakhasi	Visiting Fellow	LPP, NCI
OTHER:	Pradman K. Qasba	Visiting Scientist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of the synthesis of α-LA and casein mRNA during various stages of gestation and lactation of rat mammary gland and in rat mammary carcinomas.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the synthesis of α-LA and casein mRNA during various stages of gestation and lactation of rat mammary gland and in rat mammary carcinomas.

Methods: The methods being used are detailed in project ZO1 CB 08219-02, LPP.

Major Findings: 1) αLA and casein mRNA synthesis can be detected during various stages of gestation and reaches to 40% of the total mRNA activity. 2) During certain periods of lactation total mRNA activity is comprised by only α-LA and casein mRNA, out of which 30% is the αLA mRNA and 70% is casein mRNA.

Proposed Course of Research: 1) The levels of mRNA's will be measured also with c-DNA probes. 2) The 5' and 3' ends of αLA and casein mRNA will be investigated. 3) Precursors and processing of these mRNA will be investigated.

Significance to Cancer Research: Knowledge on the synthesis and processing of αLA and casein mRNA will help us in understanding the mechanisms involved in the expression of these differentiated functions in normal and neoplastic tissue.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08226 01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Role of Thyroid Hormones in Growth and Differentiation of Mouse Mammary Glands.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.K. Vonderhaar	Expert	LPP, NCI
OTHERS:	A. Bhattacharya	Visiting Fellow	LPP, NCI
	A.E. Greco	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: This project was designed to understand the role thyroid hormones play in growth and development of mouse mammary glands and to correlate these observations with tumor incidence. Studies include: 1) examination of the morphology of mammary glands during several developmental stages in euthyroid, hypothyroid and hyperthyroid mice; 2) evaluation of the ability of epithelial cells from these glands to differentiate in explant culture and elaborate the milk-proteins casein and α-lactalbumin under the influence of insulin, hydrocortisone, prolactin and thyroid hormones; 3) investigation of the characteristics of specific receptors for thyroid hormones and prolactin within the mammary tissue.

PHS-6040
(Rev. 10-76)

Major Objectives: The purpose of these studies was to define the morphological and biochemical development of the mammary glands from mice whose thyroid status was manipulated by chemical means. Tissue from animals showing different degrees of epithelial development due to altered thyroid status were to be examined for their ability to make the milk-proteins casein and α-lactalbumin in culture under proper hormonal stimulation. The factors influencing the coupling of this process to a round of cell division were to be defined. Furthermore, we wished to demonstrate the presence of specific receptors for thyroid hormones within the mammary gland and examine their distribution within the fat and epithelial components.

Methods Employed: C3H/HeN mice were used. Animals were rendered mildly hypothyroid by adding 0.1% thiouracil to the drinking water. Hyperthyroidism was induced by adding thyroxine (L-T_4) to the drinking water at 2 μg/ml. Morphology was examined by hematoxalin staining of whole mounted glands. An organ culture system using chemically defined serum-free medium was employed for studies on milk-protein synthesis. Casein was assayed as phosphorylated peptides using rennin -Ca++ precipitation. α-Lactalbumin was assayed for enzymatic activity in the production of lactose. Thyroid hormone receptors were assayed by standard techniques for soluble, membrane bound and nuclear forms.

Major Findings: The mammary glands of euthyroid C3H/HeN female mice undergo a series of morphological changes during development. In glands from immature animals, the epithelial component consists of a primitive ductal system which fills about one-third of the fat pad. In the adult virgin gland, the epithelial component fills the fat pad with a highly-branched ductal system and a few alveoli. During pregnancy and subsequent lactation the cells proliferate and extensive lobulo-alveolar development occurs. In contrast, glands from adult virgin animals maintained in a hypothyroid state since weaning retain the primitive ductal appearance while filling the fat pad. This mild hypothyroidism does not result in permanent hormonal imbalance as the effects of thiouracil on mammary gland development are easily reversed within 2 months of removing this chemical from the drinking water.

The simplified morphology in adult virgin hypothyroid glands does not result from deficiencies in ovarian hormones which sometimes accompany thiouracil-induced hypothyroidism. This possibility was ruled out by 3 experiments: 1) these mildly hypothyroid animals are able to become pregnant and deliver live pups; 2) the male C3H/HeN mammary rudiment which shows extensive lobulo-alveolar growth after 5 weeks of estrogen and progesterone administration to euthyroid animals, shows only primitve ducts in the hypothyroid state; 3) estrogen and progesterone administration to hypothyroid females does not alter the morphology of the mammary gland. However, extensive lobulo-alveolar development can be obtained in hypothyroid females by injecting prolactin (2 mg/day) for 2 weeks. Extensive alveolar development is also seen in glands from adult virgin hyperthyroid animals. Glands from animals made hypothyroid during 7 weeks of involution after lactation have extensive lobulo-alveolar development and are morpho-

logically indistinguishable from glands of their euthyroid counterparts.

These observations led to the conclusions that: 1) hypothyroidism induced by thiouracil arrests and retards development of the mammary epithelium but does not cause regression; and 2) epithelial growth, like α-lactalbumin induction in culture, is regulated by the ratio of prolactin to thyroid hormones.

Further studies revealed that when explants of tissue from hypothyroid animals are cultured in serum-free medium containing insulin, hydrocortisone and prolactin, the specific milk protein α-lactalbumin is induced. The level of α-lactalbumin activity induced per ng of epithelial DNA is the same as that found in explants from glands of euthyroid virgins after a similar culture period. When 10^{-9}M L-T_3 is added to either of these cultures, an additional 3- to 5-fold increase in α-lactalbumin activity is obtained.

Emergence of α-lactalbumin activity and induction of the synthesis of casein phosphoproteins in euthyroid tissue cultured in the 3-hormone system depends on DNA synthesis. Addition of L-T_3 to the cultures overcomes this dependence. When hyperthyroid tissue is cultured in the 3-hormone system, DNA synthesis in vitro is no longer necessary. This is true even when the T_4 is withdrawn from the drinking water 4 days prior to the onset of culture. In contrast to tissue from euthyroid virgins, explants from glands of euthyroid primiparous animals elaborate α-lactalbumin independent of DNA synthesis. However, the morphologically similar glands from hypothyroid primiparous mice revert to a state of dependence on DNA synthesis before induction of the milk-proteins.

These observations led to the conclusions that: 1) the ductal cells from hypothyroid virgins are fully capable of production of α-lactalbumin in culture; and 2) the coupling of differentiation to a round of cell division in the mouse mammary gland is controlled by thyroid hormones.

The ability of mammary glands from pregnant mice to bind L-T_3 was examined. Specific binding can be found in several subcellular fractions including cytosol, crude membranes and nuclei. Cytosol receptors have a Kd of $9.3 \times 10^{-10}M^{-1}$ and those in nuclei have a Kd of $6.4 \times 10^{-10}M^{-1}$. The number of binding sites in cytosol is 11.7×10^{-12} moles per mg protein. The number of sites in nuclei is 2×10^{-13} moles per μg DNA. Competition for binding can be demonstrated with thyroid hormone analogues but not with insulin, hydrocortisone or prolactin.

Cytosol from mammary fat pads at saturation bind only 2.5×10^{-13} moles T_3 per mg protein while cytosol from epithelial cells isolated from glands by treatment with collagenase bind 1.2×10^{-12} moles T_3 per mg protein.

These observations led to the conclusions that: 1) mammary glands contain specific receptors for T_3; and 2) these receptors are primarily localized in the epithelial cells of the gland.

Significance to Cancer Research:

In humans, an early age at first pregnancy decreases risk of subsequent breast cancer. By exploring fundamental differences between glands of adult virgin and primiparous animals in terms of growth response and developmental capabilities, we hope to gain insight into the possible hormonal control of this risk factor. A less clearly established risk factor in human breast cancer is altered thyroid status. Although the literature contains many conflicting observations, the bulk of evidence suggests a possible increase in risk associated with hypothyroidism. For this reason it is essential to understand the mechanism by which thyroid hormones affect growth and differentiation of mammary epithelium.

Proposed Course of Research:

We will continue to explore the differences in biochemical response between adult virgin and primiparous animals with special emphasis on those events controlled by thyroid hormones. We will attempt to demonstrate an affect of thyroid hormones on growth of euthyroid virgin mammary glands in a modified organ culture system. The mechanism of action of thyroid hormones will be explored with special emphasis on the relationship of thyroid hormones to prolaction action as reflected in their receptors. We also hope to explore these areas using the rat which may be a better model system for risk in breast cancer. The rat studies will employ animals in several thyroid states, treated with nitrosomethylurea (NMU) and will include examination of changes in incidence, latent period and number and location of tumors per rat.

Publications

1. Vonderhaar, B.K.: Studies on the Mechanism by which Thyroid Hormones Enhance α-Lactalbumin Activity in Explants from Mouse Mammary Glands. Endocrinology 100: 1423-1431, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08215-03 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Angiogenesis as a Marker of Neoplastic Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Antonino Maiorana Visiting Fellow LPP, NCI

OTHER: Pietro M. Gullino, Chief, Laboratory of Pathophysiology, NCI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous work in humans and mice indicated that about 30% of mammary epithelium was able to induce angiogenesis when transplanted on the rabbit iris. Present work aims at establishing whether (a) the rat mammary gland behaves similarly (b) the rat hyperplastic nodules with high frequency of neoplastic transformation also have higher incidence of positive angiogenic response before any sign of neoplasia is detectable, (c) whether the hyperplastic lesions of the liver behave like the mammary hyperplastic epithelium and (8) whether the angiogenesis factor is present in the interstitial fluid of the tumor in vivo.

PHS-6040
(Rev. 10-76)

Methods: Biopsy specimens of rat breast tissue are transplanted into the rabbit iris or cornea. Neovascularization is monitored with the slit-lamp stereomicroscope. The histology of the transplant is correlated to the angiogenetic capacity. Interstitial fluid of tumors is sampled with a technique previously developed.

Major Findings: (1) The majority of rat mammary carcinomas induced new vessel formation in the rabbit iris (2) the hyperplastic epithelium of the mammary gland also can induce angiogenesis but the question of frequency is not yet answered (3) the effectiveness of the iris transplantation as a method to predict "early" neoplastic changes appears good enough to be used in a routine screening of tissue fragments removed at biopsy.

Significance to Cancer Research: Previous work of Dr. M. Gimbrone (Report # NCI 08215-01) and Dr. S. Brem (Report # NCI 08215-02) established that iris heterotransplants may offer a test for establishing whether mammary epithelium which appears hyperplastic under standard histologic analysis has already acquired the ability to induce angiogenesis which is a property peculiar to neoplastic cells. Dr. A. Maiorana's work will establish how reliable the iris transplantation procedure is on a routine application, as in a screening operation, and also whether "preneoplastic" lesions in other tissues, liver in this case, behave as the mammary epithelium. Demonstration of angiogenic activity may become a test for detection of carcinomas before any histologic or other analytical procedures are predictive.

Publications

1. Brem, S.S. and Gullino, P.M.: Angiogenesis: A marker for neoplastic transformation of mammary papillary hyperplasia. Science 195: 880-882, 1977.

2. Brem, S.S., Jensen, H.M., and Gullino, P.M.: Angiogenesis as a marker of neoplastic and preneoplastic lesions of the human breast. Cancer (In press).

3. Gullino, P.M.: Natural history of breast cancer: Progression from hyperplasia to neoplasia as predicted by angiogenesis. Cancer 39:2697-2703, 1977.

4. Gullino, P.M.: In vivo release of neoplastic cells by mammary tumors. In: Cancer Metastasis: Approaches to the mechanism, prevention and treatment. P.G. Stansly and H. Sato (Eds.). Tokyo, Japan, University Press, 1977, pp. 49-55.

5. Gullino, P.M.: Considerations on the preneoplastic lesions of the mammary gland. Am. J. Pathol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08227-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Relationship between Temperature and Blood Supply or Oxygen and Glucose Consumption by Mammary Carcinomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. M. Gullino Chief, Laboratory of Pathophysiology, NCI

OTHER: F. Grantham Biological Laboratory Technician, LPP, NCI

COOPERATING UNITS (if any)

Pon-Nyong Yi, Department of Physics
De Paul University, Chicago, Illinois
Rakesh Kumar Jain, Department of Chemical Engineering, Columbia University, N.Y.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective was to determine what importance the parameters controlling heat production may have in diagnostic thermography and prognostic evaluation of tumor growth capacity. In the anesthetized animal, tumor temperature was lowered and blood supply as well as O_2 and glucose consumption were also lowered. As the temperature was raised in these tumors, a concomitant increase in blood supply and O_2 and glucose consumption was obtained. However, warming of initially normothermic tumors failed to produce any consistent change in either blood supply or O_2 and glucose consumption. Drastic alterations in circulation and blood oxygenation were necessary to appreciably (0.3°C) modify tumor temperature and the results are neither consistent nor conspicuous.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: (1) To measure directly the temperature of tumors in vivo when modifications were induced in either whole body temperature or in the tumor metabolism as influenced by blood supply and O_2 and glucose consumption. (2) To improve our understanding of the importance that parameters controlling heat production may have in diagnostic thermography and prognostic evaluation of tumor growth capacity.

Methods Employed: An experimental procedure was developed whereby a thermistor probe was incorporated by a tumor grown at the distal extremity of a vesicular folicle bearing one artery and one vein. Temperature, blood supply and O_2 and glucose comsumption were measured directly in each proportion as the environmental conditions were changed.

Major Findings: (1) The temperature of rat mammary carcinomas was higher than that of the subcutaneous tissue receiving the transplant. (2) Tumor temperature dropped during host anesthesia and the rate varied with the anesthetic. (3) When whole body temperature was changed, increment or reduction of temperature in subcutaneous tumors preceded that of the subcutaneous tissue but trailed that of the abdominal cavity. (4) In cooled tumors, blood supply and O_2 and glucose consumption were decreased and all three parameters increased upon warming. (5) Raising the temperature of initially normothermic tumors failed to produce any consistent change in blood supply or O_2 and glucose consumption. (6) Drastic alterations in circulation and blood oxygenation were necessary to appreciably (0.3°C) modify tumor temperature and the results were not reproduced consistently.

Significance to Biomedical Research and the Program of the Institute: A well-developed technology is available for detection of heat production in tissues. There are observations (Gautherie, M. et al., Functional Explorations in Senology. Golin et al., eds, European Press, 1976, pp. 93-110) linking heat generation by mammary carcinomas to their growth potential. Our work aims at improving the basic physiological knowledge on which diagnoses and prognoses of mammary carcinomas are based.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08220-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Adenylation of Basic Cellular Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. R. Stone	Visiting Fellow	LPP, NCI
Other:	W. R. Kidwell	Head, Cell Cycle Regulation Section	LPP, NCI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Modification of proteins by the covalent attachment of various small molecules such as phosphate, acetate, methyl groups, etc. occurs extensively in living organisms. These modifications serve the cell either as fine regulatory controls allowing rapid responses to external stimuli (hormones) without new enzymes protein synthesis or they are involved in regulation of protein synthesis either at the transcriptional or translational level. One such modification recently discovered in our laboratory is the adenylation of a small basic cellular protein. This project concerns the characterization of the adenylation reaction, including the identification of the modified protein and the physiological significance of the adenylation reaction in the regulation of cellular metabolism.

PHS-6040
(Rev. 10-76)

Project Description:

Methods: The adenylation reaction in intact cells is followed by means of pulsing cells with ^{3}H-adenosine followed by selective extraction of the adenylated protein with cold perchloric acid. The modified protein is then precipitated by the addition of cold TCA. Final resolution of the adenylated proteins from other contaminating radioactivity is achieved by electrophoresis of the TCA precipitated material or acid-urea polyacrylamide gels. For following the adenylation reaction in vitro, subcellular fractions are incubated with [^{3}H-adenosine]-ATP or [^{3}H-adenosine]-AMP.

Major Findings: Previous results from this laboratory have indicated that during a pulse labeling of HeLa cells with ^{3}H-adenosine, a small basic protein becomes modified by attachment of 5'-AMP. The modification of this protein by AMP does not occur in nuclei but requires cell cytoplasm. Incubation of whole cell lysates in cytoplasmic extracts with ^{3}H-ATP or ^{3}H-AMP also results in the production of the AMP-protein complex. The properties of this in vitro produced modified protein are the same as that isolated from ^{3}H-adenosine pulsed cells.

A comparison was made of the time and temperature dependency of ^{3}H-ATP and ^{3}H-AMP labeling of this protein complex in cell lysates. It is seen that in the absence of EDTA an equal amount of label is incorporated into the complex at 4°C and 10 seconds or at 25°C for 5 minutes whether ^{3}H-ATP or ^{3}H-AMP is the substrate. However, in the presence of 10mM EDTA no AMP-protein complex is produced when ^{3}H-ATP is used as substrate, whereas production of this AMP-protein complex from ^{3}H-AMP is unaffected by EDTA. We have shown that ATP is rapidly converted to AMP and ADP in cell lysates and that in the presence of EDTA, AMP production from ATP is blocked. Thus it is clear that AMP is the immediate substrate for complex formation and that ATP must first be converted to AMP.

All of this complex-forming activity is in a 105,000 x g supernatant fraction of HeLa cells and rat liver when subcellular fractions are isolated and incubated with ^{3}H-AMP. Also, this AMP-protein complex is formed in vivo in cultured cells as well as in the livers and mammary tumors of perfused rats.

Significance to Biomedical Research and the Program of the Institute: (NCP Objective #6, Approach #3). Understanding the basic control mechanisms of cellular metabolism is essential for the successful design of ways to reimpose growth regulation on cancer cells whose normal controls are lost in the neoplastic state.

Proposed Course: A major effort will be made to isolate the subcellular fraction which contains the adenylated protein and to isolate and characterize the protein.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08221-02 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of Histone H1 Dimer Poly(ADP-Ribose) Complex Formation by Poly(ADP-Ribose) Glycohydrolase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wishard S. Lorimer, III	Research Associate	LPP, NCI
OTHERS:	William R. Kidwell	Head, Cell Cycle Regulation Section	LPP, NCI
	Peter R. Stone	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: Poly ADP-ribosylation of basic chromosomal proteins has been reported to occur both in vivo and in vitro. Functionally, this modification may present a mechanism for disrupting the electrostatic attraction between positively charged basic proteins and negatively charged chromosomal DNA. The reaction is thus likely involved in altering the degree of chromatin condensation during processes such as DNA and RNA synthesis, DNA repair or in preparation for mitosis. The process of poly ADP-ribosylation of chromosomal proteins is most likely a reversible one since poly(ADP-ribose) turns over rapidly via an enzyme, poly(ADP-ribose) glycohydrolase, present in chromatin.

The objectives of this project are twofold:

(1) to purify and characterize the activity of poly(ADP-ribose) glycohydrolase; and

(2) to correlate the differences in the relative amounts of enzyme activity present in normal versus neoplastic tissue.

PHS-6040
(Rev. 10-76)

Methods: The synthesis and turnover of poly(ADP-ribose) in normal and transformed human and rat cell nuclei was assessed as follows. Nuclei were purified from cell or tissue homogenates by sucrose density gradient centrifugation. Chromosomal proteins were then poly ADP-ribosylated by the endogenous poly(ADP-ribose) polymerase utilizing ^{3}H-NAD as substrate. Turnover of the polymer via endogenous poly(ADP-ribose) glycohydrolase was analyzed by following the time course of the decrease of acid precipitable radioactivity following a block of further poly(ADP-ribose) synthesis by addition of an inhibitor of the polymerase to the incubation mixture. Comparisons of the endogenous levels of poly(ADP-ribose) glycohydrolase and the extent of chromosomal protein modification were evaluated by electrophoretic analysis of the mobility of poly ADP-ribosylated H1 histone on acid urea or SDS-urea gels. Alternatively, poly ADP-ribosylated H1 was digested with glycohydrolase purified from calf thymus, then electrophoresed.

Major Findings: The electrophoretic mobilities of H1-poly(ADP-ribose) complexes in human mammary cells (HBL-100 and MCF-7) and in normal lactating mammary tissue, normal liver or mammary tumors of rats were compared. The R_f of the histone-polymer complex from the human cell nuclei was exactly the same as the protein-polymer complex formed in Hela cell nuclei. This latter species has been found to consist of 2 H1 molecules linked by a single chain of poly(ADP-ribose) (P.R. Stone, this laboratory). Thus the complex formed in all the human cells appears to be H1 dimerized by a polymer bridge. In contrast, nuclei from normal lactating mammary gland made complexes which electrophoresed with unmodified H1 monomer as well as H1 dimer. The complex formed in normal rat liver or in rat mammary tumors (MTW-9) electrophoresed nearly exclusively with H1 monomer. Analysis of the average polymer chain length in the complex indicated an inverse relationship existed between polymer size and electrophoretic mobility of the complex. The polymer chain length made in the nuclei was found to relate inversely with the amount of endogenous poly(ADP-ribose) glycohydrolase in the nuclear preparations. These observations suggest that a crosslinking of H1 molecules occurs via chains of poly(ADP-ribose) and that the linking process is reversed or prevented by hydrolysis of the polymer by the endogenous glycohydrolase.

Thus it is clear that chromatin protein modification by polymer attachment can be controlled by polymer degradation. Whether changes in this catabolic process are regulated by changes in amount of glycohydrolase protein or by activators or inhibitors of this enzyme has been evaluated by characterizing the glycohydrolase purified from calf thymus according to Miwa (J. Biol. Chem. 249, 1974). The enzyme has been freed of contaminating phosphodiesterases and nucleases. It is a basic protein with a pI of about 10. It acts exoglycosidically degrading the polymer from the non-reducing end of the chain. In vivo, enzyme activity may be regulated by the level in the nucleus since it is inhibited by ADPR, cAMP, and single stranded DNA.

Significance for Cancer Research: (NCP objective #6, Approach #3) Understanding the mechanisms whereby nuclear processes, such as chromatin condensation are controlled during cell growth may suggest ways in which

cancer cells can be reversed to a nontransformed state or killed. The results presented suggest that poly(ADP-ribose) serves a role in modifying the structure of the chromosomal fiber by acting as a bridge between proximal folds of the fiber thus acting as a local transient fixative. Poly(ADP-ribose) glycohydrolase seems to control the degree to which this bridging occurs. Such a process appears to be essential to the progression of the cell to division since inhibitors of polymer synthesis leads to cell death.

Proposed Course of Research: Attempts will be made to purify and develop antibodies to poly(ADP-ribose) glycohydrolase. Then small changes in the concentration of this enzyme in tissue extracts or possibly even serum could be monitored.

Studies are currently underway to assess the role of poly(ADP-ribose) glycohydrolase in DNA repair mechanisms.

Publications

1. Stone, P.R., Lorimer, III, W.S. and Kidwell, W.R. Proceedings of the 4th International Symposium on Poly(ADP-Ribose) and ADP-Ribosylation of Proteins. (Ed.,H. Hilf) Walter de Gruyter, Berlin, N.Y., 1976, p.6.

2. Lorimer, III, W.S., Stone, P.R., and Kidwell, W.R. Control of Histone H1 Dimer - Poly(ADP-Ribose) Complex Formation by Poly(ADP-Ribose) Glycohydrolase. Experimental Cell Research (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05211-06 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modification of Histone H1 by Poly(ADPR) in Hela Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. R. Stone	Visiting Fellow	LPP	NCI
Other:	W. S. Lorimer, III	Research Associate	LPP	NCI
	W. R. Kidwell	Head, Cell Cycle Regulation	LPP	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous experiments have revealed that nuclei of eukaryotic cells contain an enzyme which polymerises the ADP-ribose moiety of NAD through a 1'-2'-ribose-ribose linkage to form poly(ADP-ribose). The polymer is at least in part associated with basic chromosomal proteins and in particular histone H1. The current objective is to characterize the nuclear proteins to which poly(ADP-ribose) is attached with special emphasis on histone H1 and to elucidate the significance of this protein - poly(ADP-ribose) association in so far as nuclear processes during cellular growth is concerned.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed: Nuclei or chromatin is isolated from HeLa cells and incubated with ^{3}H-NAD to produce poly ADP-ribosylated nuclear proteins. Selective extraction of the H1-associated poly(ADP-ribose) was achieved by virtue of the differential solubility of H1 in 5% PCA and its insolubility in 18% TCA. Subsequent analysis of the H1 preparation was performed using SDS polyacrylamide and acid-urea polyacrylamide gel electrophoresis as well as ion exchange chromatography.

Major Findings: Chromosomal poly(ADP-ribose) polymerase attaches polymer chains to H1 histone. Examination of the electrophoretic properties of H1 histone extracted from HeLa cell nuclei incubated with NAD has revealed the presence of a protein species with a slower mobility than H1. When NAD was omitted from the incubated nuclei, this protein band was absent from the gels. Analysis of the gels for radioactivity incorporated from ^{3}H-NAD indicated that the poly(ADP)-ribose) synthesized was almost exclusively associated with the slower moving band rather than with H1. When poly(ADP-ribose) was dissociated from the protein by NaOH or NH_2OH before electrophoresis, the slower migrating protein band was absent from the gels.

A kinetic examination of the formation of the H1 associated protein-poly(ADP-ribose) complex in vitro revealed an inverse relationship between the amount of histone H1 and the amount of poly ADP-ribosylated protein complex detectable by gel electrophoresis. This indicated that the polymer-protein complex was derived by ADP-ribosylation of Histone H1. Also, the labeling ratio of ^{14}C-arginine to ^{3}H-lysine is identical in both H1 and the polymer-protein complex indicating H1 to be the protein component of the complex.

A Poly(ADP-ribose) chain serves as a bridge linking H1 molecules to form histone dimers. The average chain length of the poly(ADPR) in the complex has been determined independently by three different methods: hydroxylapatite column chromatography, digestion with snake venom phosphodiesterase and finally by digestion with poly(ADP-ribose) glycohydrolase purified from calf thymus. A consistent value of 15 ADP-ribose units has been obtained.

The mass of histone H1 present in the complex in acid-urea gels has been independently determined by two methods. One method relies on the amino acid analysis of ^{3}H-lysine labeled H1-polymer preparations while the second method relies on H1 quantitation by staining of the protein band on acid-urea gels. From the mass of histone H1 and the amount of polymer in the complex (calculated from the specific activity of the substrate ^{3}H-NAD and the ^{3}H-polymer cpm in the complex on the gels) the stoichiometry of H1 to polymer in the complex has been determined to be 7-8 ADP-ribose residues for each H1 molecule. Since the chain length of the polymer is 15, this means that the complex is composed of two H1 molecules in association with one molecule of poly(ADP-ribose) - a polymer - H1 dimer. Only one end of the polymer seems to be covalently attached to H1 and that is the reducing

end of the polymer which is associated with the N-terminal half of the histone H1 molecules.

Individual conformational changes of chromatin selectively promote H1 dimer formation. Addition of spermine to the nuclei incubated with ^{3}H-NAD results in little stimulation of the total acid-insoluble polymer synthesized but enhances production of poly(ADP-ribose)-H1 dimer. This probably results from a spermine induced conformational change in the chromatin affecting both the poly(ADP-ribose) polymerase activity and the accessibility of substrate H1 molecules.

Significance to Biomedical Research and the Program of the Institute: (NCP Objective #6, Approach #3). Understanding the mechanisms whereby nuclear processes, such as chromatin condensation are controlled during cell growth may suggest ways in which cancer cells can be reversed to a non-transformed state or killed. The results presented suggest that poly(ADP-ribose) serves a role in modifying the structure of the chromosomal fiber by acting as a bridge between proximal folds of the fiber thus acting as a local transient fixative. Such a process appears to be essential to progression of the cell to division since inhibitors of polymer synthesis leads to cell death.

Proposed Course: One major task is to isolate sufficient quantity of highly purified polymer-H1 dimer to enable the characterization of this species with regard to such properties as the site and mode of attachment of the polymer on the H1. The effect and role of agents, which cause conformational changes in the chromatin (e.g., spermine), will be studied on the extent of dimer synthesis in an attempt to elucidate the functional role of the H1-dimer in the nucleus and its significance in cellular growth.

Publications:

Stone, P. R., Lorimer, W. S. III, and Kidwell, W. R.: Proceedings of the 4th International Symposium on Poly(ADP-ribose) and ADP-ribosylation of Proteins. In Hilz, H. (Ed.), Walter De Gruyter, Berlin, N.Y., 1976, p.6.

Hilz, H. and Stone, P. R.: Poly(ADP-Ribose) and ADP-ribosylation of proteins. Rev. Physiology, Biochemistry and Pharmacology 76: 1-58, 1976.

Lorimer, W. S. III, Stone, P. R., and Kidwell, W. R.: Control of Histone H1 dimer - Poly(ADP-ribose) Complex Formation by Poly(ADP-ribose) Glycohydrolase. Expt'l Cell Res. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08229-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Dietary Lipids in Increased Incidence of Mammary Cancer.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William R. Kidwell, Head, Cell Cycle Regulation Section LPP, NCI

OTHERS: Marie Monaco, Consulting Expert, Medical Oncology, NCI
Marc Lippman, Senior Investigator, Medical Oncology, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Pathophysiology

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Cell Cycle Regulation Section

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NCI, NIH, Bethesda, Maryland 20016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary of Work: Epidemiological surveys have shown an increased incidence of mammary cancer in recent years, especially in Japan. This increased incidence is positively correlated with increased consumption of animal fat. Experimentally, dietary lipids, especially unsaturated fatty acids, have been shown to enhance the efficiency of carcinogen induced rat and mouse mammary tumors and to promote the growth of transplantable mammary tumors in mice. Our objective is to determine the mechanism by which the process occurs - either directly on mammary epithelial cells with lipids acting as initiators or promoters or as nutritional factors or, alternatively, whether lipids promote the process indirectly on mammary cells by influencing the hormonal status of the host. The approach is to evaluate the above alternatives by comparing the effects of lipids on normal rodent mammary epithelial cell growth and transformation as well as the nutritional requirements for growth of cultures derived from carcinogen induced tumors.

PHS-6040
(Rev. 10-76)

Methods: Cells were isolated from DMBA induced, primary rat mammary tumors by collagenase digestion and propogated in growth median supplemented with 10% fetal calf serum and 5% serum from adult, cycling female rats of same strain. The cells were established as clones by growth from single cells in micro wells. De novo lipid synthesis was assessed by the incorporation of ^{3}H-acetate. Fatty acids were quantitated spectrophotometrically following conversion to their respective p-bromophenacyl esters and separation by high pressure liquid chromatography. Effects of lipids on cell growth were performed by adding pure lipids to cells plated in media containing serum from which lipids were removed by organic solvent extraction.

Major Results: From primary DMBA induced rat mammary tumors, a clonal cell line has been established. The cells appear to be derived from the epithelial compartment of the tumor since (1) they respond to lactogenic hormone, (2) they grow in media in which D-valine is substituted for L-valine and (3) they have an epithelioid appearance. Other mammary epithelial cell markers (casein, lactose synthetase) were not detectable. The cells are not normal epithelial cells since they have a hypotetraploid chromosome number. Attempts to date to obtain tumors by innoculation into isologous hosts has not been successful.

Hormonal Responsiveness: These cells resemble explants of mouse mammary gland in that they respond to insulin plus prolactin giving up to a 35 fold stimulation of acetate incorporation into fatty acids. Insulin alone stimulates but the insulin effect is increased from 3-5 fold by prolactin. Comparison of the type of fatty acid synthesized with or without added hormones indicates no preferential stimulation of short chain fatty acids such as is observed in explant cultures of late pregnant or lactating mammary gland. Rather, the percent distribution of ^{3}H-acetate label in the various fatty acids is nearly identical to that of lipids synthesized in virgin mammary gland explants. Fatty acid synthesis is not promoted by estradiol or progesterone. Growth hormone could replace prolactin in the assay but human placental lactogen was inactive.

Lipid Effects on Cell Growth: Rat serum was found to stimulate growth of the cultures by a factor of 2-3 compared to fetal calf serum. Analysis of the two sera revealed that rat serum has 18 times as much linoleic acid as fetal calf serum. Other fatty acids were present at 1-2 fold higher concentrations. At least half of the effect of rat serum on cell growth was ascribed to its content of unsaturated fatty acids since direct addition of purified fatty acids enhanced growth rate. Saturated fatty acids were nearly totally inactive in this regard. Unsaturated fatty acids ranked in order of decreasing growth stimulating potential were: linoleic acid > arachidonic acid > oleic acid > linolenic acid.

These results raise the possibility that dietary unsaturated fatty acids may lead to an apparent increased incidence of mammary tumors by promoting tumor growth rate thus facilitating tumor detection.

Lipid Effects on Uptake of ^{14}C-DMBA into Mammary Cells: Addition of free fatty acids to cells grown in lipid free medium led to a dose dependent accumulation of lipid droplets in the cells' cytoplasm. Intracellular accumulation of the carcinogen, 7,12-Dimethylbenzanthracene correlated positively with the size of the accumulated lipid droplets. However, linoleic acid was 2-5 times more effective in promoting DMBA uptake as well as DMBA retention by the cells than was an equal concentration of palmitic acid. Thus dietary unsaturated fatty acids are more effective in both growth promotion and in carcinogen uptake than saturated fatty acids in the mammary tumor cells which we have isolated.

Significance for Cancer Research: (NCP objective #1, Approach 3). Two aspects of lipid effects on cells were detected. Unsaturated fatty acids, particularly linoleic acid were shown to be more effective than saturated fatty acids in growth promotion and in carcinogen uptake by mammary tumor cells in culture. Both aspects may be important in explaining the increased incidence of mammary cancer which correlates epidemiologically with increased consumption of animal fats.

Proposed Course of Research: The studies to date have been conducted with a single cell line established from rat mammary tumors. To determine whether the results obtained are generally applicable, the studies will be extended to a variety of established human and rodent cell lines and to primary cultures of normal mammary cells from experimental animals. These latter cultures will also be examined for the possibility that lipids may act as co-carcinogens in the transformation process. Additionally, dietary lipids will be evaluated for their effects on endogenous hormone levels, particularly those hormones known to affect mammary cell growth.

Publications

1. Kidwell, W.R. and Mage, M.G. Changes in poly(ADP-ribose) and poly (ADP-ribose) polymerase in synchronous Hela cells. Biochem. 15: 1213-1217, 1976.

2. Kidwell, W.R. Radioimmunoassay of poly(ADP-ribose) in extracts of growth-synchronized Hela cells. In Hilz, H. (Ed.): Proc. 4th Internat. Symp. on poly(ADP-ribose) and ADP-ribosylation of protein. Berlin, Walter de Gruyter, 1976, p.8.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08210-03 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Behavior of Ribosomes, Polysomes and Ribosomal Subunits in Non-dividing Human Cells and Changes Related to Onset and Cessation of Normal Cell Growth.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Herbert L. Cooper, Head, Cellular and Molecular Physiology Section, LPP, NCI

OTHERS: Mats Harms-Ringdahl, Visiting Fellow LPP, NCI

Richard Braverman, Chemist LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
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SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: To characterize the behavior of ribosomal particles in a non-growing cell population in which the rate of protein synthesis is maintained at low levels; to correlate such behavior with the limitation in protein synthesis so as to understand the mode of translational control employed by the cell in adjusting protein synthesis to growth and metabolic activities; to determine the regulatory steps which are operative in the enhancement of protein synthesis associated with the onset of cell growth; to detect and characterize the biochemical steps which control protein synthesis during the physiological cessation of cell growth and division.

PHS-6040
(Rev. 10-76)

Methods: Purification of lymphocytes from human peripheral blood by combined nylon column adsorption and density sedimentation. Preparation of cytoplasmic extracts for density gradient analysis of subcellular particle distributions. Extraction and purification of RNA from intact cells and from cell fractions, analyzed by density gradient, electrophoretic, gel filtration and chromatographic techniques. Chromatographic and cell fractionation techniques for separation and purification of cell proteins (dissociation factor). Direct and indirect assay techniques for determining dissociation factor activity in lymphocytes (described below). Quantitation of specific tRNA levels by measurement of aminoacylation with radioactive amino acids. Preparation of enucleated cells (cytoplasts) by treatment with cytochalasin-B followed by density-gradient sedimentation. Cell disruption and fractionation by nitrogen cavitation. Other general laboratory, cell culture and radiolabeling techniques.

Major Findings: 1) We have previously shown that over 70% of the ribosomes in resting lymphocytes are free ribosomes which do not participate in protein synthesis. On growth stimulation with phytohemagglutinin virtually all of these particles are activated to enter protein synthesis. Quantitation of the amount of new rRNA and protein synthesis reveals that the increase in protein synthesis occurring in the initial 10 hrs after PHA treatment is entirely dependent on the mobilization of pre-existing free ribosomes and cannot be accomplished with newly synthesized ribosomes. Since the early increase in protein synthesis by stimulated lymphocytes includes all of the proteins which are essential to the normal immunological activity of lymphocytes, the large pool of inactive ribosomes carried by resting lymphocytes may be seen as a physiological adaptation which is essential for the rapid increase of protein synthesis required by differentiated cell functioning.

Free ribosomes were found to re-accumulate after growth stimulation, at the peak of cell growth and protein synthesis. This apparent paradox may be understood when it is realized that, after a period of cell growth (both *in vivo* and *in vitro*) lymphocytes return to the resting state where they are again susceptible to stimulation. The accumulation of free ribosomes during cell growth may represent preparation for the state of quiescence which will follow. This indicates that regulatory mechanisms relating cell growth and protein synthesis are often of an anticipatory nature, providing for future contingencies.

2) The mechanisms regulating the participation of ribosomes in protein synthesis are under study. From the kinetics of formation of free ribosomes we concluded that new ribosomal subunits enter the cytoplasm, participate in protein synthesis for 15-20 minutes, then are inactivated as free ribosomes. On the basis of functional and physical measurements of ribosomal particles it was concluded that ribosomes were inactivated because they failed to dissociate into subunits at the completion of a round of protein synthesis. Since dissociated subunits are required for initiation, failure to dissociate prevents return to protein synthesis. Ribosome dissociation has been shown, in other systems, to be

controlled by a protein, dissociation factor (DF). The existence of DF in lymphocytes was verified after partial purification of the protein and assay of its ability to dissociate free ribosomes. We are currently adapting a method for assaying DF activity which is based on the ability of dissociated 60S subunits to catalyze peptide bond formation between puromycin and N-blocked amino acids. This requires the synthesis of acetyl [^{3}H] phenylalanyl-tRNA and accumulation of large numbers of 80s free ribosomes as reagents. When this technique is perfected, it will enable us to test the hypothesis that an early step in the activation of free ribosomes, and hence of protein synthesis, is an increase in DF. Measurements of DF activity throughout the growth cycle will be made. Preliminary evidence suggests that a certain amount of DF is present in an inactive form in resting lymphocytes, and that this may be activated early in growth stimulation. If this is confirmed, the mechanism of activation will be studied.

3) Mobilization of free ribosomes to form polysomes requires not only dissociation into subunits, but also, participation of those subunits in the initiation steps of translation. We have obtained information about the control of initiation through experiments in which RNA synthesis is inhibited with actinomycin-D. It was found that no stimulation of protein synthesis or formation of polysomes occurred when PHA was given to lymphocytes treated with actinomycin-D. Pre-existing polysomes dissappeared and protein synthesis decreased with 1st order kinetics and a half life of about 1.5h. in resting or growing lymphocytes. Thus, continued or increased protein synthesis required continued RNA synthesis. The site at which Actinomycin interfered with protein synthesis was found to be prior to the formation of the 40s initiation complex. The only RNA involved in formation of 40s initiation complexes is $tRNA_f^{met}$, the initiator tRNA. Evidently, protein synthesis cannot be stimulated in lymphocytes in the absence of tRNA synthesis, and this material becomes rate-limiting on protein synthesis in resting cells shortly after its synthesis is halted. Detailed kinetic studies show that the rate of protein synthesis begins to decline immediately after tRNA synthesis is halted, which may indicate that this is the rate-limiting component for protein synthesis in resting cells. Measurements of the amount of $tRNA_f^{met}$ in resting lymphocytes showed that there was only 1 molecule for each 5 native subunit pairs in the resting cell, and that this value rose during PHA stimulation. Our current working hypothesis is that the rate of protein synthesis is controlled by the level of $tRNA_f^{met}$ in resting lymphocytes, and that lymphocyte activation must involve an early increase in the synthesis of this material.

4) The above interpretation of experiments using actinomycin-D depends upon being certain that the drug affects only nuclear RNA synthesis, and does not directly interfere with formation of the 40s initiation complex. This was shown to be true by experiments in which lymphocytes were enucleated by cytochalasin-B treatment. Such "cytoplasts" continue to synthesize protein for a number of hours. Protein synthesis in cytoplasts from either resting or growing lymphocytes was completely unaffected by actinomycin under the same conditions as those in which protein synthesis was markedly inhibited in intact cells. Further, PHA had no

effect on protein synthesis when added to resting-cell cytoplasts. Thus actinomycin-D does not interfere directly with initiation, and PHA stimulation requires nuclear participation to elevate protein synthesis.

5) Studies were carried out during extended culture of lymphocytes stimulated with concanavalin-A, to follow events related to cessation of cell growth. During the period 48-72 hrs, when cell growth and DNA synthesis were at their height, a dissociation between the rates of RNA and protein synthesis was observed, indicating a relative slowing of protein production. This occurred despite increasing numbers of polysomes. Our tentative explanation of this finding is that during this period, "arrested" polysomes occur, which do not complete protein synthesis. During this same period, new ribosomal subunits appear to enter the free ribosome pool before participating in protein synthesis, suggesting that they enter the cytoplasm without DF. These notions will be further explored. The studies have identified a period in cell growth when preparations for cessation of growth may be occurring.

Significance to Cancer Research: By the elucidation of the mechanisms which regulate protein synthesis in resting cells and during a physiological growth sequence, we hope to provide the basis for recognition and understanding of derangements in these controls which may characterize malignant transformation. This objective supports Objective 3-1 of the NCP.

Proposed Course of Research:

1) Development of assay for dissociation factor and application to determine whether changes in DF levels correlate with changes in free ribosome pool.

2) Study of mechanism by which DF activity is increased by growth stimulation.

3) Extension of studies to determine whether protein synthesis in lymphocytes is regulated by $tRNA_f^{met}$ content.

4) Study of mechanism by which $tRNA_f^{met}$ production is increased by mitogen stimulation.

5) Continuation of studies of the controls involved in cessation of cell growth.

Publications

1. Cooper, H.L., Berger, S., Braverman, R.: Free Ribosomes in Physiologically Nondividing Cells: Human Peripheral Lymphocytes. J. Biol. Chem. 251: 4891, 1976.

2. Cooper, H. L. and Braverman, R.: Free Ribosomes and Growth Stimulation in Human Peripheral Lymphocytes: Activation of Free Ribosomes as an Essential Event in Growth Induction. J. Cellular Physiol. (In press)

3. Cooper, H.L.: Ribosome Utilization and Regulation of Protein Synthesis During Lymphocyte Activation. Proceedings of XI Leucocyte Culture Conference (D. Lucas, Ed.) Academic Press, N.Y. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08212-03 LPP (consolidated with 08209)

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis, Utilization and Degradation of Messenger RNA in Human Lymphocytes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. L. Berger Research Chemist LPP, NCI

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cellular & Molecular Physiology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The control of messenger RNA metabolism will be studied in human lymphocytes from the point at which mRNA precursors in the nucleus can be distinguished from bulk hnRNA, to the destruction of mature mRNA molecules in the cytoplasm. In vivo, the steps involved include control of transcripton of hnRNA, selection of molecules for subsequent polyadenylation, transport of designated poly(A)$^+$ as well as poly(A)$^-$ components to the cytoplasm and the relationship between protein synthesis and the survival of particular mRNAs. Experiments to devise methods for inhibiting or activating these processes in order to manipulate cell growth are anticipated. The studies will encompass normal, resting and growing cells as well as cultured lines of lymphoid origin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the steps involved in the synthesis and degradation of messenger RNA and its precursors; to determine the mechanisms employed by the cell in exercising control at key steps in mRNA metabolism and to devise methods of inhibiting or activating these processes in order to manipulate cell growth.

Methods: Routine procedures have been used throughout.

Major Findings: There is now a large body of data implicating heterogeneous nuclear RNA as the precursor for mRNA. However, despite several structural features in common, such as methylated GTP caps at the 5' end, internal O-methyl groups and tracts of polyadenylate at the 3' end, it is clear that a large amount of hnRNA is degraded in the nucleus, serving no known function.

The kinetic relationships between mRNA and its putative precursor, hnRNA, have been examined in the resting lymphocyte. From the results of pulse-chase experiments, 2 classes of hnRNA$^+$poly(A) have been distinguished. The first class is synthesized mainly with exogeneous label supplied during the pulse. A minimum of 40% of the polyadenylated hnRNA molecules in this class contribute to mature labile and stable mRNA$^+$poly(A). The second class of hnRNA$^+$poly(A) is synthesized predominantly from reutilized precursors during the chase incubation. In order to determine the amount of reutilization actually occurring, a calculation was made based on the apparent half-life of hnRNA$^+$poly(A) and the true half-life. The latter value is estimated from the decay of pulse-labeled molecules in the presence of actinomycin D. From the computation, it was found that lymphocytes, chase incubated for 7 hours with a high concentration of nonradioactive precursors, produced 4-fold more labeled hnRNA$^+$poly(A) during the chase than they made previously during a 6 hr pulse. This "chase labeled" material is not precursor to mRNA. Late in the chase when newly radiolabeled hnRNA$^+$poly(A) is present in abundance, no labeled mRNA$^+$poly(A) is transported to the cytoplasm.

The fate of very short-lived and stable mRNA$^+$poly(A) in the cytoplasm was then examined in detail in resting cells. After a pulse of radioactivity in which both components were labeled, 50% of the radioactive molecules were not associated with the protein synthesizing apparatus of the cell. The unbound material, probably in association with protein, had a sedimentation coefficient of approximately 10S - 40S and a size distribution, when deproteinized, similar to that of bulk mRNA$^+$poly(A) in the cytoplasm. Most of the remaining, newly synthesized mRNA$^+$poly(A) was found associated with polysomes; a small portion cosedimented with dissociated ribosomal subunits.

Pulse labeled cells were then incubated under chase conditions in order to determine the subsequent fate of the unbound poly(A)-bearing RNA. A large amount disappeared, consistent with the known degradation of very short-lived mRNA. The material remaining after the chase consisted entirely of the stable component of polyadenylated mRNA. Approximately half of the stable

mRNA was polysome-associated; the other half was divided almost equally between unbound material (10-40S) and material cosedimenting with, but not necessarily bound to, dissociated ribosomal subunits. The non-polysome associated stable mRNA was not stored material, often referred to as "masked message". It is in equilibrium wtih the mRNA being translated.

The function of labile message was not made clear by any of these techniques because it survives for such a short interval. Although it is not possible to demonstrate its association with polysomes during a chase incubation, the possibility remained that labile message was translated one or more times, before being degraded. To ascertain whether or not resting cells could translate labile mRNA, techniques were employed to drive mRNA onto polysomes, artificially. Cells which have been treated with cycloheximide and pactamycin display an increase in monosomes when compared with untreated cells. The drugs prevent translocation of ribosomes already bound to messenger RNA and trap additional mRNA as 80S initiation complexes. The production of new monosomes depends upon an excess of active ribosomal subunits, called native subunits, initiator methionyl tRNA, and all other factors required for forming the complex. By means of this approach, labeled, unbound stable mRNA^{+}poly(A) could be forced onto monosomes but unbound, labile molecules could not, despite the fact that most of the labeled mRNA in the cell was both labile and unbound. The additional monosomes produced made use of stable, unlabeled messenger RNA.

The function of non-translated, very short-lived mRNA in resting cells remains obscure. However, an examination of the life times of mRNAs in normal mitogen stimulated lymphocytes and in a malignant cultured cell line designated as MOLT-4 has led to new insights. Normal growing lymphocytes do not contain labile mRNA. Using pulse-chase techniques similar to those used in studying the survival of mRNA^{+}poly(A) in resting cells, a value of 45 hours was found for the half-life of poly(A)-bearing mRNA, after 18 hours of mitogen stimulation. In these cells, which have not yet entered S phase, only one class was identified. Drugs that prevented either new RNA synthesis or polyadenylation of preexisting mRNA also failed to uncover multiple classes of mRNA although the survival of these molecules with time differed in the drug treated cultures. Short-lived mRNA was also absent from dividing lymphocytes which had progressed further in the growth sequence. The absence of labile mRNA from normal growing cells together with the presence of untranslated, short-lived mRNA in resting cells suggests that this RNA is stabilized soon after growth stimulation and codes for growth related functions. Such a scheme would allow the resting cell to respond to a changing environment without the need for an immediate increase in mRNA synthesis.

In MOLT-4 cells, mRNA^{+}poly(A) decays with a half-life of 2 hours in the presence of actinomycin D. Once again, there is only a single class. This decay rate is much faster than that found under the same conditions in normal growing cells. Since MOLT cells resemble neither resting nor growing normal lymphocytes, is is tempting to speculate that the rapid turnover of mRNA in cultured cells represents an abberation related to the malignant state.

Publications:

Berger, S. L.: Growth of Specific Cells: Human Lymphocytes. In Methods in Enzymology: Tissue Culture Jakoby, W.B. and Pastan, I., eds. Academic Press, New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08211-03 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Detection of Specific Messenger RNAs in Resting and Growing Human Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert L. Cooper	Head, Cellular and Molecular Physiology,	LPP,NCI
OTHERS:	Shelby L. Berger	Research Chemist	LPP, NCI
	Richard Braverman	Chemist	LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cellular and Molecular Physiology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Discontinued for the present time.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08225 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Structure and Function of Biological Membranes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva, Head Membrane Biology Section		LPP, NCI
OTHERS:	A. Peixoto de Menezes	Visiting Fellow	LPP, NCI
	K. Shimizu	Visiting Scientist	I, LID
	R. Hendler	Head, Membrane Biology Sect.	LCB,NHLB
	B. Berghoffer	Bio. Lab. Technician	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

COOPERATING UNITS (if any)
M. L. Nogueira and S. Bartnicki-Garcia, Department of Plant Pathology, University of California, Riverside, California

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES (Blood) ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Comparative morphology of the plasma membranes from mammary tissue, normal and cancerous. 2) Mechanisms of fusion of plasma membranes. 3) Morphology of Schwann cell membranes; study of axolemma/Schwann cell relationships. 4) Membrane events during milk fat secretion by lactating mammary glands. 5) Cell wall formation during encystment of Phytophthora palmivora zoospores. 5) Interaction of virus with erthyrocyte membranes studied by scanning electron microscopy. 7) Freeze-fracture morphology of membranes reconstituted from lipid and protein components isolated from mitochondria.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The elucidation of the structure and function of biological membranes. The understanding of the aspect of membrane biology which may differentiate normal from cancer cells.

Methods: Appropriate cells are fixed in glutaraldehyde, impregnated in glycerol and rapidly frozen in the liquid phase of partially solidified Freon 22. Specimens are freeze-fractured in a Balzers device and a platinum-carbon replica case of the fractured faces is made. Morphology of the replica is studied with the electron microscope for scanning microscopy, fixed tissues are subject to critical point drying, coated with gold and observed.

Major Findings: Based on electron microscopic observations of P. palmivora zoospores during encystment we were able to demonstrate many of the steps in membrane reorganization which occur during fusion. We have proposed a new theory for membrane fusion events, especially those involved in secretory processes. (See publication No.1). We also found that in P. Palmivora zoospores of the cyst cell wall is not accompanied by visible restructuring of the plasma membrane (see publication no. 3).

Analysis of the freeze-fracture morphology of the mechanism of milk fat secretion of lactating mammary gland cells allows new views for the mechanism of formation of the milk fat globule membrane. (manuscript in preparation). The study of rat sciatic nerve shows the presence, in internodal regions, of structural specializations which probably represent sites of interaction of the axollema with the periaxonal portions of Schwann cell membranes. These findings may be significant in analysis of the process of nerve conduction (see publication No. 2).

Significance to Cancer Research: Membrane fusion is a frequent event in cells. Among others, it mediates most secretory mechanisms as well as many mechanisms of viral infection of cells. We hope that our findings and their interpretations will provide the basis for a general mechanism of membrane fusion.

Membranes bear out their outer surface most or all of the chemical groups that provide the characteristics of all surfaces. Cell surfaces are directly responsible for many of the membrane interactions. It is thought that the altered social behavior of cancer cells reflects, in large measure, altered characteristics of their cell surface and, consequently, of their plasma membranes. Thus, the study of the structure and function of biological membranes is at the heart of the understanding, of the basic biology of cancer cells. The study of the structure and function of biological membranes is, at present, at an early phase. It is, thus, necessary when determining the priority of research problems, to choose those that present technology as well as scientific knowledge makes potentially more attractive. For these reasons, research programs centered around cancerological aspects have to devote considerable effort into areas of membrane biology which are not immediately connected to cancer. Basic membrane biology is, in this way, one of the research areas included

in the programs of many of the laboratories at the National Institutes of Health.

Proposed Course: Future research will involve the study of the membrane biology of mammary gland epithelial cells as well as virus-plasma membrane interactions. Other areas of membrane biology will be pursued including the structure of membranes reconstituted from bacterial lipid and protein components.

Publications:

Pinto da Silva, P. and Nogueira, M. L.: Membrane Fusion during Secretion. A Hypothesis Based on Electron Microscopic Observation of Phytophthora palmivora Zoospores During Encystment. J. Cell Biol. 73: 161-181, 1977.

Miller, R. and Pinto da Silva, P.: Particle Rosettes in the Periaxonal Schwann Cell Membrane and Particle Clusters in the Axollema of Rat Sciatic Nerve. Brain Res. (in press).

Nogueira, M. L., Pinto da Silva, P. and Bartnicki-Garcia, S.: Freeze-fracture and Freeze-etching Study of Encysting Phytophothora palmivora Zoospores. J. Gen. Microbiol. (in press).

Pinto da Silva, P.: Freeze-fracture and Freeze-etching: Possibilities and Limitations. Revista de Microscopia Electronica. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00380-21 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Deoxyribose Containing Compounds in Normal and Cancer Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W.C. Schneider Head, Nucleic Acids Section LPP, NCI

COOPERATING UNITS (if any)
R. Gantt, LB, NCI; G. H. Smith, LMBGY, NCI; N. Wivel, LCBGY, NCI;
S. Chattapodhyay, PO, NCI

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: This program's objectives are to isolate and identify deoxyribose-containing compounds other than DNA from normal and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal, precancerous, and cancer tissues, to isolate DNA from subcellular structures other than nuclei, to study the physical and biochemical properties of extranuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Since the replication of DNA is an obligatory prerequisite for cell division, the study of DNA synthesis and its control occupies a position of unique importance in cancer research due to the continuous and uncontrolled division occuring in cancer tissues. The objectives of this project are to isolate and identify deoxyribose-containing compounds other than DNA from normal, precancerous, and tumor tissues, to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal, precancerous, and cancer tissues; to isolate DNA from subcellular structures other than nuclei, and to study the physical and biochemical properties of extranuclear DNA and to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

These studies are aimed at uncovering differences between normal and malignant cells that can be exploited to prevent the formation of cancer cells or to promote the destruction of cancer cells already generated. (These studies support most closely Objective 3: To prevent conversion of cells to those capable of forming cancer and Approach 1: Nature and modification of the precancerous state, of the National Cancer Plan).

Methods Employed: Nuclei, mitochondria, zymogen granules, and microsomes were isolated from rat or mouse tissues by differential and/or density gradient centrifugation as well as by centrifugation in the zonal rotor. Nucleic acids were extracted from tissue fractions using sodium lauryl sulfate (SLS) and phenol. DNA was purified from these extracts by banding in CsCl, by chromatography on hydroxylapatite, or by treatment with NaOH to remove RNA and small molecules.

DNA samples were examined by means of analytical centrifugation, melting behavior, and amount of labeled thymidine incorporated. DNA was measured by a modification of the diphenylamine reaction or by the fluorescense produced in the reaction of Kissane and Robins. Enzyme markers were used for estimating purity of isolated subcellular fractions.

The reassociation kinetics of DNA were measured on hydroxylapatite columns using phosphate buffers of prescribed concentrations for elution of denatured and reassociated DNA (Britten, Methods in Enzymology). The DNA samples used in these studies were either sheared in a high speed blender or treated with acid and alkali to obtain smaller fragments.

DNA samples were treated with restriction nucleases and the products were separated by gel electrophoresis and visualised with ethidium bromide.

Major Findings: During the past year, two aspects of the program on cytoplasmic DNA have been completed and the results have been published or are in press. In addition, other work has been extended and new approaches designed to test whether microsomal DNA is a unique DNA have been instituted.

Reassociation kinetics of liver DNA. Studies on the renaturation of nuclear, mitochondrial and microsomal DNA have been completed and published. Briefly, the results showed that the three types of DNA could be readily differentiated by their reassociation kinetics. Mitochondrial DNA renatured rapidly ($C_o t_{1/2}$ = 0.03) while microsomal DNA reassociated more slowly ($C_o t_{1/2}$ = 0.14) and nuclear DNA reassociated over a broad range of $C_o t$ values ($C_o t_{1/2}$ = 500). These studies provide strong evidence that microsomal DNA like mitochondrial DNA is a unique type of DNA.

Zymogen granules (with Dr. Smith). In order to determine whether DNA was also associated with zymogen granules, a particulate organelle found in certain secretory glands, a method had to be devised to obtain these granules in highly purified form. The use of the zonal ultracentrifuge rotor permitted the isolation of these granules from rat pancreas in a single step and in good yield and high purity as judged by electron microscopy and assays for marker enzymes. The analysis of the isolated zymogen granules revealed that they contained small amounts of DNA as well as RNA. These findings are now in press in Analytical Biochemistry. The results with regard to DNA have been extended by demonstrating that the zymogen DNA could be banded in Cs Cl thus further securing its identification as DNA. In an effort to differentiate zymogen DNA from other cellular DNA samples, incorporation of ^{3}H-thymidine was measured in one experiment. The radioactivity of the zymogen DNA was found to be lower than that of mitochondrial DNA but the results will have to be repeated under conditions yielding more incorporation and more DNA.

New approaches to differentiation of cytoplasmic DNA. Although good evidence is now available for the uniqueness of both mitochondrial and microsomal DNA, additional experiments have been undertaken to strengthen these conclusions and to offer additional methods by which other DNA samples, such as zymogen DNA, could be characterized.

With Dr. Gantt, studies are underway to differentiate DNA samples with restriction nucleases. These enzymes split DNA at specific base sequences and yield fragments that separate as distinctive bands on gel electrophoresis. Preliminary experiments have yielded band patterns for mitochondrial and microsomal DNA samples. Since nuclear DNA samples give no evidence of bands, the results would indicate that cytoplasmic DNA samples are unique.

In another approach with Drs. Wivel and Smith, mammary tumor tissue cultures are being enucleated with cytochalasin and the cytoplasts examined for DNA associated with mitochondria and microsomes using biochemical fractionation procedures and autoradiography. These experiments have not yet yielded definitive results.

In a third approach with Dr. Chattapadhyay, cytoplasmic DNA samples are being examined by hybridization techniques for viral DNA sequences that have already been established to be present in nuclear DNA using ^{3}H-DNA probes prepared from purified virus preparations. These experiments are still in their initial stages but should yield a clear cut indication as to whether microsomal DNA originates in the nucleus.

Significance to Biomedical Research and the Program of the Institute: The experiments described above have provided strong additional evidence for considering microsomal DNA as a unique form of DNA and that there may be DNA in still other organelles such as zymogen granules. The functions of cytoplasmic DNA remain undetermined but the fact that cytoplasmic DNA represents such a small percentage of the total cellular DNA could mean that the effect of a carcinogen might have a greater impact in the cytoplasm than in the nucleus. The probability of the interaction of carcinogens with cytoplasmic DNA rather than with nuclear DNA would be greater because of the proximity of the cytoplasm to the external environment. Also, since many chemical carcinogens are converted to more active forms enzymatically in the cytoplasm, the possibility of interaction with cytoplasmic DNA would appear even more likely. For these reasons, the continued study of cytoplasmic DNA is deemed to be of great importance.

Proposed Course: The study of cytoplasmic DNA will be continued along lines already initiated with greatest emphasis on studies of DNA in cytoplasts and zymogen granules, the effect of restriction nucleases on DNA samples, and the occurrence of viral sequences in cytoplasmic DNA.

Publications:

Schneider, W. C.: Cytoplasmic DNA: Differentiation by Reassociation Kinetics. Biochem. Biophys. Res. Comm. 74; 1607-1612, 1977.

Schneider, W. C. and Smith, G. H.: On the isolation of zymogen granules from rat pancreas by zonal ultracentrifugation and their nucleic acid content. Analytical Biochem. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08228-01 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Control of Synthesis of Serum Albumin and the Metabolism of Albumin Messenger RNA (mRNAalb.) in Normal Liver and Hepatic Carcinoma.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pran K. Chakrabartty	Visiting Fellow	LPP, NCI
OTHER:	W.C. Schneider, Head, Nucleic Acid Section,		LPP, NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acid Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objective: The long range objective of this project is to examine the regulatory events involved in the synthesis of serum albumin. The translational control and post-translational processing will receive specific attention and the role/s of hormones/s, if any, in the synthesis of the protein will be studied. The effects of various carcinogenic chemicals in the synthesis of albumin and mRNAalb. metabolism will be tested. The relationship between nuclear, extra-nuclear DNA and mRNAalb. will be sought.

PHS-6040
(Rev. 10-76)

Project Description: Malignant cells undergo continuous uncontrolled cell division. DNA synthesis and protein synthesis are prerequisites for this process to continue. Because of the complexity of eukaryotic DNA it is necessary to have specific biochemical markers to study the regulation/s at the various steps of cell division. Regulation of mRNAalb. can be used as a specific indicator for that purpose. The objective of this project is to isolate mRNAalb. for the generation of complementary DNA (cDNA) to use in the study of involvement of mRNAalb. in normal, precancerous and cancerous tissue.

Methods Employed:

(1) Isolation of RNA from liver and selection of mRNA by affinity chromatography.

(2) Preparation of *in vitro* protein biosynthesizing system using sheat germ S-30 fraction and identification of specific protein by immunoprecipitation.

(3) Increasing the level of mRNAalb. *in vivo* by treatment with a drug-trioacetamide (TAA).

Major Findings: mRNA was isolated from livers of untreated normal and TAA treated animals and was efficiently translated in the wheat germ cell free system.

The amount of mRNAalb. was indirectly quantitated by immunoprecipitation of enzymatically synthesized albumin with antialbumin serum. It was found that about 10% of the mRNA in liver is mRNAalb. A two-fold increase in mRNAalb. was obtained after treatment of the animals with TAA.

Significance for Cancer Research: By the use of albumin cDNA probe the levels of mRNAalb. can be measured precisely in normal, precancerous and malignant tissue. The treatment with TAA, a liver carcinogen, indicate that an increase in the level of mRNAalb. may bear a relationship with the onset of cancer in the liver. The validity of a theory that the microsomal DNA may be "messenger" DNA can also be tested using the probe or directly using the mRNA. The establishment of a biological role of microsomal DNA, if any, in normal and cancerous tissue will be of paramount importance.

Proposed Course of Research:

(1) Purification of mRNAalb.

(2) Synthesis of cDNA probe using mRNAalb. template.

(3) Molceular hybridization with liver microsomal DNA to access its biological role as "messenger" DNA.

(4) Test of the level of mRNAalb. in various hepatic carcinoma cells.

Publications

1. Charkrabartty, P.K. and Qasba, P.K.: Partial Purification of Rat α-lactalbumin mRNA. Nucleic Acids Res. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05205-10 LPP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Biochemical Mechanisms for Control of Nucleic Acid Synthesis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth P. Anderson	Research Biochemist	LPP, NCI
OTHER:	Alec S. Liacouras	Research Biochemist	LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Pathophysiology

S.

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

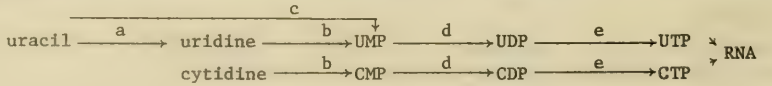
CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: The long-range objective of this project is to investigate nucleotide metabolism in normal and cancer cells. The aim is to elucidate pathways of biosynthesis, mechanisms of the enzymatic reactions involved, mechanisms of control over these reactions and pathways, and biochemical mechanisms of sensitivity and resistance to purine and pyrimidine analogs. The end-products of nucleotide biosynthesis are the triphosphate precursors of nucleic acids, and regulation of the enzymes involved in these biosynthetic steps is presumably a major factor in determining the rate of cellular nucleic acid synthesis. In view of the central role of UMP in pyrimidine metabolism, present work is focused on reactions b and d in the following pathway of pyrimidine nucleotide biosynthesis:



Project Description:

Major Findings: 1) Studies on uridine-cytidine kinase. In mammalian tissues uridine-cytidine kinase (reacton b above) is the initial step in pyrimidine nucleotide biosynthesis from the preformed ring. This reaction has been shown to be rate-limiting in the biosynthetic pathway and susceptible to feedback inhibition by nucleoside triphosphate end-products (Anderson and Brockman, Biochim. Biophys. Acta 91: 380, 1965). We have now purified this enzyme from the mouse ascites mast cell neoplasm P815/S, in which we first demonstrated feedback regulation of the enzyme (Liacouras and Anderson, Arch. Biochem. Biophys. 168: 66, 1975), and also from the mouse ascites 29P hepatoma. The final preparation from P815/S has a specific activity of 3.02 μ/mg, the most highly purified preparation of this enzyme yet isolated, representing 1800-fold purification from the whole tumor tissue. Sensitivity to the feedback inhibition by the triphosphate end-products CTP and UTP is fully retained by the enzyme throughout the purification. We have characterized the purified enzyme with regard to a number of parameters (Liacouras, Garvey, Millar and Anderson, Arch. Biochem. Biophys. 168: 74, 1975). The enzyme exhibits a broad specificity for the phosphate donor substrate, but is much more specific for the phosphate acceptor, with only uridine, cytidine, and their analogs serving as substrates. Thus, 5-fluorouridine and the 5-aza- and 6-aza- analogs of uridine and cytidine are phosphorylated bythe enzyme, but thymine riboside, the deoxyribonucleosides, and cytosine arabinoside are not phosphorylated. Using the purified enzyme, we have also shown that the initial velocity pattern for the phosphorylation of either uridine or cytidine is one of intersecting lines rather than parallel lines, indicating that the reaction proceeds by the sequential addition of both substrates (ATP and nucleoside) to the enzyme to form a ternary complex, followed by the sequential release of the two products (nucleoside monophosphate and ADP). A ping-pong mechanism, in which the product of the first half-reaction is released prior to the addition of the second substrate to the enzyme, is ruled out by this initial velocity pattern. Analysis of the data by Cleland's Sequen computer program indicated a K_m of the enzyme for uridine of 1.5×10^{-5} M, and for ATP, with either uridine or cytidine as phosphate acceptor, of 3.6×10^{-3} M or 2.1×10^{-3} M, respectively. The V_{max} was 1.83 μmoles phosphorylated/min/mg enzyme protein for the uridine kinase reaction and 0.91 μmole for the cytidine kinase reaction. The kinetic studies and the analysis of the reaction mechanism have been published (Arch. Biochem. Biophys. 168 74, 1975). Further kinetic studies have now indicated that, with either uridine or cytidine as substrate, the pattern of inhibition by the immediate product CMP is non-competitive with respect to the nucleoside substrate. A random sequential addition of substrates would result in competitive inhibition patterns for either product against either substrate. Hence, the non-competitive inhibition pattern produced by CMP against the nucleoside substrate suggests that the sequential addition of substrates to the enzyme may be ordered rather than random.

Other kinetic studies have now explored the competition between the two alternative nucleoside substrates, uridine and cytidine, to delineate whether both are phosphorylated by a single enzyme at the same catalytic site or whether the two phosphorylations are mediated by separate isoenzymes that purified together. The theory of the method used has been described by Cha (Mol.

Pharmacol. 4: 621, 1968), and the patterns obtained are determined by the V_{max} and K_m values of the two alternative substrates. The method plots the combined velocity against the concentration of one substrate at increasing fixed concentrations of the alternate substrate, and a family of curves is obtained. When the velocity is plotted against the concentration of the better substrate (higher V_{max}, uridine) at different fixed concentrations of the poorer substrate (lower V_{max}, cytidine), the curves intersect at a point where the poorer substrate appears to have no effect on the reaction velocity. The data we have obtained are in good agreement with Cha's theoretical analysis of competition between two alternate substrates reacting at a single catalytic site. The intersection poin was shown to occur around 013 - 0.35 mM uridine. When the combined velocity is plotted against the concentration of the poorer substrate at different fixed concnetrations of the better substrate, teh curves do not intersect but rather converge toward the V_{max} of the poorer substrate. Again the actual data obtained closely fit the theory. It can therefore be concluded that the phosphorylation of both uridine and cytidine is catalyzed by a single enzyme protein and a single catalytic site, rather than by two separate isoenzymes, each specific for one nucleoside and one nucleoside triphosphate feedback inhibitor. This work is now in press in Molecular and Cellular Biochemistry.

Alternate-substrate competition studies have now also been carried out with either uridine or cytidine and either the cytidine analog, 5-azacytidine, or the uridine analog, 5-fluorouridine. The studies with azacytidine have been done by Dr. Liacouras; those with fluorouridine have been carried out by Mr. Free. We have found the K_m and V_{max} for 5-azacytidine to be 8.6×10^{-3} M and 2.1 µmoles phosphorylated/min/mg protein, prespectively, in experiments with enzyme preparations exhibiting a V_{max} of 4.2 µmoles/min/mg with uridine and 2.2 with cytidine. Thus the V_{max} for azacytidine was half that for uridine and equal to that for cytidine. When uridine and azacytidine were studied in combination, and 1/combined velocity was plotted against 1/uridineconcentration, at several fixed concentrations of azacytidine, a family of curves was obtained with an intersection near 0.25 mM uridine. The pattern resembled that for uridine plus cytidine, so again the data agree with the theoretical for two substrates competing at the same enzyme site. If the same data were plotted as 1/combined velocity against 1/5-azacytidine concentration, at the several concentrations of uridine, the resulting curves did not intersect, but merged toward $1/V_{max}$ for azacytidine, again in agreement with the theoretical. In contrast with these patterns, for alternate substrates with equal V_{max} values, the plot of 1/combined velocity against the reciprocal of either substrate concentration gives a series of curves which merge toward the V_{max} value for either substrate alone (Cha, Mol. Pharmacol.). Thus neither substrate inhibits the reaction velocity of the alternative substrate. Azacytidine and cytidine have equivalent V_{max} values, and experiments with combinations of the two substrates gave patterns which, again, were in good agreement with the theoretical prediction. The experiments with 5-fluorouridine indicated a K_m of 2.0×10^{-4} M and a V_{max} of 2.1 µmoles/min/mg with uridine and 1.4 with cytidine. Thus, the V_{max} for fluorouridine was euuivalent to that for uridine and greater than that for cytidine. Again the patterns observed in combined substrate

experiments resembled the theoretical patterns for substrates competing at the same enzymatic site. In the experiments with uridine plus fluorouridine, the family of curves for 1/combined velocity plotted against 1/[S] did not intersect but merged toward the common V_{max} value. In the experiments with cytidine plus fluorouridine, 1/combined velocity plotted against 1/cytidine concentration (the poorer substrate) gave a family of curves which did not intersect but merged toward the V_{max} for cytidine. The same data plotted against 1/fluorouridine concentration (the better substrate) gave a family of curves which intersected near 0.33 mM fluorouridine. The data indicate, therefore, that the same enzyme which phosphorylates uridine and cytidine also catalyzes the reaction with the analogs 5-azacytidine and 5-fluorouridine, and presumably at the same active site. The work on 5-azacytidine was presented at the Federation meetings in Chicago in April, 1977; both studies are presently being prepared for publication.

In recent explorations of other possible substrates for the kinase, reaction has been tested with 3-deazauridine, pyrazomycin, and pseudouridine. Both of the former compounds have attracted recent clinical interest as anticancer agents and both presumably might function as phosphorylated derivatives. The studies have shown that pyrazomycin is not a significant substrate, but that it does inhibit the phosphorylation of uridine, apparently noncompetitively. In contrast, both 3-deazauridine and pseudouridine were phosphorylated. The K_m's were 5.4×10^{-5}M for deazauridine and 1.75×10^{-2}M for pseudouridine; V_{max} values were 2.46 and 3.28 μmoles phosphorylated/min/mg protein, respectively. It is known that after incorporation of uridine into RNA, pseudouridine is enzymatically synthesized through a break of the N--C bond from the base to the ribose moiety, followed by a rotation of the uridine ring to form a C--C bond. The present evidence now suggests that a second biosynthetic pathway for pseudouridine incorporation into RNA by way of the triphosphate may exist. Although the K_m is high, the reaction velocity indicates that the compound may be considered a reasonable substrate for uridine-cytidine kinase.

Our purification of uridine-cytidine kinase and the results of our several years of characterization of the enzyme are, by invitation, also currently in press in Methods in Enzymology in a forthcoming volume on "Purine and Pyrimidine Nucleotide Metabolism".

2) Studies on UMP-CMP kinase. We have previously purified UMP-CMP kinase 300-fold from Tetrahymena pyriformis (Ruffner and Anderson, J. Biol. Chem. 244: 5994, 1969) and defined its reaction mechanism (Garvey, Millar, and Anderson, Biochim. Biophys. Acta 302: 38, 1973); Tetrahymena pyriformis is a rapidly growing protozoan that is unable to synthesize pyrimidine or purine rings de novo and hence must depend on anabolism from the preformed ring structures by reactions a, b, and c in the scheme above. UMP is then the central compound in pyrimidine nucleotide metabolism and the anabolism of this compound into the triphosphate precursors of nucleic acids is related to the rapid synthesis of nucleic acids during active growth. Our purification and characterization of UMP-CMP kinase is also, by invitation, currently in press in Methods in Enzymology in the forthcoming volume on

"Purine and Pyrimidine Metabolism".

Significance for Cancer Research and the Program of the Institute: UMP is the central compound in pyrimidine nucleotide biosynthesis in that it is the precursor for synthesis of other pyrimidine nucleotides of both the ribo- and deoxyribo- series. UMP synthesis by uridine kinase is called into play in rapidly growing tissues such as tumor tissues, and this constitutes a difference between normal and cancer cells. It is important to discover how the enzymes for UMP synthesis and metabolism are regulated in both types of cells, since such regulation is crucial in maintaining a balance among the levels of the various nucleotide precursors of nucleic acids and functions in regulating the rate of cellular nucleic acid synthesis. (NCP Objective 3, Approach 2).

Proposed Course of Research:

Mr. Free has transferred to another institute. He will have two publications forthcoming from the present studies, one of which is currently in preparation.

Dr. Liacouras is moving to a project on melanin DNA, With Dr. Walter Schneider. He will have three more publications forthcoming from the present studies, one of which is currently in preparation.

Since many of the answers sought with regard to the reaction mechanism and characterization of these purified enzymes ahve been found, Dr. Anderson will be extending the studies on pyrimidine nucleotide metabolism into other areas and exploring possible isoenzyme patterns as potential probes to monitor hyperplastic and neoplastic stages in the neoplastic transformation of mammary tissue.

Publications

1. Liacouras, A.S. and Anderson, E.P.: Uridine-cytidine Kinase III. Competition between Uridine and Cytidine for a Single Enzyme. Molecular and Cellular Biochemistry, 1977 (In press).

2. Anderson, E.P.: Uridine-cytidine Kinase from a Murine Neoplasm. Methods in Enzymology. Purine and Pyrimidine Nucleotide Metabolism. 1977 (In press).

3. Anderson, E.P.: UMP-CMP Kinase from Tetrahymena Pyriformis. Methods in Enzymology. Purine and Pyrimidine Nucleotide Metabolism. 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00306-14 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modification of Amino Acids in Search of Possible Anti-tumor Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. T. Otani Research Chemist LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: To prepare new amino acids and amino acid analogs and their derivatives by organic chemical methods in search of compounds that destroy cancer cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To prepare chemical compounds which preferentially destroy cancer cells by mechanisms other than those of agents presently being used in cancer chemotherapy. Our studies with the amino acid analog, β-hydorxy-norleucine, prepared in this Laboratory, have shown that acylation of certain amino acid analogs "activated" these compounds to inhibit the growth of certain microorganisms where no such activity was observable with the free amino acid.

The immediate objectives of the project have been:

(a) To prepare various acyl derivatives of other amino acids and amino acid analogs and to note if these compounds exhibit similar microbial growth-inhibitory capacities.

(b) To prepare other new amino acids analogs, which by their structural resemblance to essential natural amino acids, may be potential antimetabolic and, hence, growth-inhibitory agents.

(c) To prepare derivatives of these amino acids to contain organic functional groups which may potentiate the growth-inhibitory as well as increase the cell permeability capacities of the molecules.

(d) To test these new compounds, for anti-tumor activity, first in microbial anti-tumor screening system, and if found active, and in mammalian systems when obtained in sufficient quantities.

Methods Employed: The β-hydroxyamino acids were prepared by methods developed in this Laboratory. An appropriate aldehyde is condensed with glycine in the presence of catalytic amounts of cupric ions (or with glycine copper complex) in a basic medium under conditions which are empirically determined. Diastereoisomers were separated by partition column chromatography. Optical isomers were resolved by asymmetric enzymatic hydrolysis using hog renal acylase and bovine carboxypeptidase. Acylation was accomplished by the Shotten-Baumann reaction. Microbiological assays were carried out as described for riboflavin assay, but with minimal quantities of the vitamin added to give maximum growth.

Major Findings:

(1) The study with 25 N-chloroacetyl amino acids, prepared in this Laboratory, showed that, in general, where there was no activity noted with the free amino acid, many of these when chloroacetylated, showed significant activity.

(2) It was noted that not all chloroacetyl derivatives showed this activity, but, in general, only those of amino acids considered as essential and those of analogs of essential amino acids.

(3) N-Acetyl and N-propionyl derivatives were also prepared and tested for growth-inhibitory activity in the Lactobacillus casei system. Neither the N-acetyl nor the N-propionyl derivative showed any activity.

(4) Hence, in general, it appears that both the nature of the acyl and of amino acid moieties were important in the activity of the acyl amino acid as an inhibitor.

(5) The study has been extended to include N-trifluoroacetyl and N-benzoyl derivatives. Twenty five N-trifluoroacetyl and 15 N-benzoyl derivatives of amino acids and of amino acid analogs were prepared.

(6) In addition, numerous free amino acids, N-acetyl- and N-benzoyl-amino acids obtained commercially were extensively purified by recrystallization and by passage through cationic exchange resin. The purity of the compounds prepared in our Laboratory and those obtained commercially was checked by (a) melting point determination, (b) paper chromatography, (c) optical rotation determination, (d) Van Slyke nitrous acid determination of primary amino nitrogen, and (e) elemental analysis.

(7) Thus far, 12 N-trifluoroacetyl derivatives have been tested for growth-inhibitory activity against the L. casei system. It was found that while the acetyl and the propionyl derivatives showed no activity, some of these showed moderate, but significant, activity.

(8) In some cases, where the free amino acid is a potent cytotoxic agent, when aceytlated, it became completely innocuous, showing a protective effect of the acyl group.

(9) Control experiments indicated that the inhibition exhibited by the chloroacetyl amino acids is not likely due to the non-enzymatic release of chloroacetic acid, itself a potent inhibitor of this system.

(10) A certain preparation of chloroacetyl-L-tyrosine showed marked inhibition in the L. casei system showing complete inhibition of growth at a low concentration.

(11) Preliminary tests to identify the substance, including melting point determination, optical rotation determination, release of constituent amino acid by acid hydrolysis and chromatographic identification of the free amino acid, Van Slyke nitrous acid determination of primary amino nitrogen, elemental analysis, nuclear magnetic resonance spectra, and infra-red absorption spectra have indicated that the inhibitory substance is a contaminant, and is present in minute quantities.

(12) Subsequent to observing marked inhibitory activity with the N-chloroacetyl-L-tyrosine, analogs of tyrosine were obtained, and the corresponding N-chloroacetyl derivatives were synthesized. The method of preparation of these compounds was similar to that used for the previous acyl

compounds, except that in certain cases, additional precautions were taken to prevent acylation of other susceptible groups, and to prevent deacylation of acyl groups already present in the molecule. The purity of these compounds was checked by similar procedures as described for the other amino acids and acyl derivatives (above).

(13) So far, growth-inhibitory studies of these compounds using the L. casei system showed, as in the previous cases, that while no appreciable activity was noted with the free amino acids, these amino acids when chloroacetylated, exhibited significant inhibitory activity.

Significance to Biomedical Research and the Program of the Institute: That amino acid metabolism may be a susceptible area of action in cancer therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Amino acid deprivation, either by the removal of the amino acid or by the substitution with an analog may be effective in inhibiting tumor growth.

The unnatural amino acids synthesized under this program are close structural analogs of essential natural amino acids, differing in some cases only in the presence of a single hydroxyl group adjacent to the α-amino group. Hence, β-hydroxynorleucine differs from norleucine only in the presence of a hydroxyl group in the β-position of the carbon chain of the amino acid. β-Hydroxynorleucine is a structural analog of methionine, differing from the latter in having a methylene group in place of the sulfur atom in the γ-position of the chain. Indeed, β-hydroxynorleucine, even as a mixture of four isomers, exhibited inhibitory action in two microbial systems. β-Hydroxymethoxinine and β-hydroxymethionine are even closer analogs of methionine than is β-hydroxyhomomethionine, the former differing from methionine in possessing an oxygen atom in place of the sulfur atom and in possessing a β-hydroxyl group, and the latter only possessing a β-hydroxyl group.

The chain-halogenated amino acids, similarly, are close structural analogs of essential amino acids. For example, γ-dichlorothreonine would be an analog of leucine, since the size of the chlorine atom is approximately that of the methyl group; also γ-trifluorothreonine would be a close analog of threonine since the atomic radius of the fluorine atom is essentially that of hydrogen atom.

The enhancement of activity of amino acid, and amino acid analogs upon acylation appears to be of importance in the cancer program in view of the fact that one serious drawback in cancer chemotherapy has been that of the inaccessibility of the cytotoxic agent to the cell. It is possible that alteration of the molecular charge by acylation, and the introduction of a cytotoxic group, in the form of the chloroacetyl radical, has provided a molecule that is more permeable to the cell and at the same time, once within the cell, capable of releasing the cytotoxic agents by the action of intracellular acylases. Objective 6, Approach 2.

Proposed Course:

(1) To continue comparative studies of the effect on the growth-inhibiting activity of other acyl groups attached to the amino acids and amino acid analogs --- the N-trichloroacetyl- and the N-benzoyl-amino acids.

(2) To prepare the N-trifluoroacetyl- and N-benzoyl-derivatives of β-hydroxynorleucine, the N-chloroacetyl derivative of which showed notable degree of inhibition.

(3) To prepare halogenated derivatives of threonine and of leucine, and from these, their N-chloroacetyl derivatives.

(4) To prepare the γ-trifluoro analog of threonine and from it, the N-chloroacetyl derivative.

(5) To prepare non-amino acid derivatives of these amino acids.

(6) To prepare analogs of tryptophan, i.e., β-hydroxytryptophan, from indole-3-carboxyaldehyde and glycine-copper complex.

(7) To test all of these compounds thus prepared for anti-tumor action, first in a microbial anti-tumor pre-screen system, and then in a mammalian tumor system when the compound is found to be active or when obtained in sufficient quantities.

(8) To develop a model system for testing these agents using protozoa.

Publications:

Otani, T. T. and Briley, M. R.: B-Hydroxynorleucine: Separation of its isomers and biological studies. J. Pharm. Sci. 65: 534-537, 1976.

Otani, T. T. and Briley, M. R.: The effect of acylated amino acids and acylated amino acid analogs on microbial anti-tumor screen. J. Pharm. Sci. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05221-04 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Comparison of nucleoproteins of normal animal tissues with neoplastic tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Rotherham Research Chemist LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To discover if there are changes in nucleoprotein structure that are basic to neoplastic change and to characterize them.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To discover if there are differences in the structures, properties and metabolism of the nucleic acids and nucleoproteins of normal rat and/or mouse tissues with respect to comparable tissues that are becoming neoplastic.

Methods Employed: Suitable chemical agents will be used which enhance the occurrence of neoplastic changes in mouse and/or rat tissues. Nuclear fractions will be isolated and examined for possible differences in metabolic modifications of the nucleoproteins of tissues of treated and control animals.

Major Findings: Beyond that given in the report on this project for July 1, 1974 through June 30, 1975, no new data have been obtained.

Significance for Cancer Research: (See Objectives) National Cancer Program Plan Objective and Approach: Cause and prevention: Nature and rate of oncogenic cell transformations in carcinogenesis (3,2).

Proposed Course of Research: Work has continued on this project in the directions indicated in previous reports. The method used for isolating nuclei from mouse epidermis has been improved now to the extent that the nuclear preparations are sufficiently clean to be used as starting material for extraction of nuclear proteins. In photomicrographs of stained preparations of isolated nuclei the contamination with cytoplasmic material was seen to be low. Nuclear proteins isolated from these nuclear preparations were purified and analyzed by electrophoresis on polyacrylamide gels. All of the bands that would be anticipated to be present in normal tissue were seen. In regions of the gels where it would have been expected that protein contaminants of cytoplasmic origin would be located, no bands were seen.

Since March 1, experimental work on this project has come to a halt pending action on requests for necessary qualified technical assistance and for equipment for measurement of radioactivity. The matter of technical assistance is a first priority to resuming the laboratory work and within a few weeks following the equipment would be needed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00919-14 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cytochemical Features in Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. C. Reid Supervisory Research Chemist LPP, NCI

COOPERATING UNITS (if any)

Quantitative Cytology Section, LP, DCBD

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: New techniques being developed for automatic cytology require improved markers for differentiating peculiar cells. This project aims to evaluate the feasibility of using the surface/mass ratio as a characteristic parameter.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Development of technique for characterizing abnormal cells suitable for automatic systems.

Methodology: Physicochemical methods for characterizing macromolecular and topologic features of cell surfaces.

Major Findings:

1. During the past few years there has appeared an increasing flow of reports from electron microscopists showing that many types of cells exhibit an elaborate surface morphology very different from the smooth membrane assumed classically. Diverse filaments, lamella, blebs and pores appear and some workers feel that cells transformed carcinogenically exhibit characteristic changes in this surface morphology, although data are still rather scanty.

In some cells the surface structures are extensive, which suggests that the surface/mass ratio may be enhanced sufficiently to make this ratio a useful parameter for distinguishing different types of cells. I have made some rough calculations based upon filament dimensions estimated from published photographs which indicate that differences of 2-3 fold might be expected and the values can be considerably higher under optimistic assumptions.

A practical test would require the use of a marker which would stain only the exterior cell surface, presumably macromolecular since most of the claims for surface specificity of small molecules are disputed. In addition, the stain should bind covalently. It appears that there is no such agent available at present and an effort is being made to formulate some chemical approaches.

2. Various activities peripheral to outside contracts may have been useful in resolving chemical problems. These would be reflected in the report of the quantitative cytology group.

Significance for Cancer Research: Present activities relate to the development of technique for mass screening by automatic systems. Objective 5, Approach 4.

Proposed Course: An attempt will be made to formulate a practical approach to the utilization of surface/mass ratio as a parameter for distinguishing diverse types of cells present in disperse mixtures and possibly in aggregates.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00944(a)-15 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Total Metabolism of Cancer Cachexia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. D. Morrison Research Physiologist LPP, NCI

COOPERATING UNITS (if any)

Laboratory of Theoretical Biology, NCI

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Energy Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: This project is directed towards identifying the causes of the nutritional depletion and general deterioration of the host, known as cancer cachexia, with a view to blocking or reversing these systemic-effects of cancer so that the cancer patient could become more accessible to anti-cancer therapies. The approach to this is by investigation of total energy, water and other material exchanges, and of the physiological and behavioral control of food and water intake in normal and cancerous organisms.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

(a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth, to finding the nature of the loads imposed by a tumor on its host, and to identifying the causes of cancer cachexia.

(b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth.

(c) Examination of possible experimental methods of simulating in non-tumor-bearing rats the cachexia and decline of food intake induced by tumor growth.

(d) Identification of functional sites and causes of breakdown of control of food intake during tumor growth.

(e) Development of conceptual models of control of food and water intake and regulation of energy and water exchange and their interrelationships for normal animals and for the cancer cachectic process.

(f) Development of quantitative and reproducible assessment of behavioral output, to yield more sensitive and earlier detection of systemic effects of tumors.

(g) Advisory and administrative activities to enable effective development and prosecution of the Diet, Nutrition and Cancer Program.

(h) Other activities.

Methods Employed: The methods of indirect, total, long-term calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Ballistic methods of recording and partitioning total motor activity. Computer methods of numerical analysis of serial records of tumor growth, continuous records of gaseous exchange and of change in feeding and drinking patterns in relation to total energy exchange and tumor growth. Sprague-Dawley rats and Buffalo rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

Major Findings:

(a and b) Since the completed installation and testing of the data acquisition and processing equipment (referred to in the Annual Report for 1976) five 3-month studies of feeding patterns in relation to tumor growth, taste aversion and energy exchange have been completed experimentally. Analysis of these is proceeding. Some preliminary findings concerning energy metabolic constraints on feeding will be reported to the Physiological Society (U.K.) in June 1977.

(c and d) Normal control of feeding is a complex and highly redundant system. Individual components of the system can be deleted or can be innately absent without grossly interfering with overall control (Morrison, S. D., Am. J. Physiol. 224: 1305, 1973; Cancer Res. 35: 3339, 1975). Feeding responses to different stimuli (e.g. change in nutrient density, change in environmental temperature, exogenous insulin, taste adulteration, etc.) represent different overlapping components of the control system. Previous work (Morrison, S. D., Cancer Res. 32: 968, 1972; Cancer Res. 33: 526, 1973) showed that response to nutrient density is immediately and progressively impaired by tumor growth. Responses to environmental temperature change and to exogenous insulin are also distorted and/or attenuated at subsequent stages of tumor growth: studies on these responses are in advanced progress but are still incomplete: preliminary aspects of this work have been reported (Morrison, S. D., Cancer Res. 35: 3339, 1975). Studies have been started on the response to 2-deoxy glucose (a competitive glucose analogue). It seems likely that cachectic hypophagia develops as a consequence of successive deletion of different components of feeding control until the redundancy of the normal control complex is lost and catastrophic collapse of control and of feeding ensues (Morrison, S. D., Physiol. Behav. 17: 705, 1976). With loss, this year, of half of the laboratory space necessary to continue this work, some of it is now being carried on in a less sophisticated way, under outside contract. This includes all the work on environmental temperature response and, in addition, studies in reactivity of tumor bearers to taste aversion. These studies are in progress.

(e) Work is progressing on the development of conceptual models of control of food intake that would correct errors and misconceptions of existing models (Morrison, S. D., Nature 255: 169, 1975) and that would allow analysis and inclusion of observed changes in feeding during tumor growth. Computer analysis of long-period records of meal patterns and of energy expenditure is being developed in collaboration with Dr. F. K. Millar (Laboratory of Theoretical Biology, DCBD).

(f) The possible modes of change in feeding that can produce change in food intake (Morrison, S. D. and Coffey, N. F., J. Appl. Physiol. 34: 268, 1973) are being examined in relation to cachectic hypophagia. It has been shown that, for most of tumor growth, a decline in one mode (change in amount of feeding activity) is fully compensated by increase in another mode (change in efficiency of feeding) with no resultant change in food intake at

this stage (Morrison, S. D., Cancer Res. 36: 228, 1976). This indicates that the cachectic process starts very early in tumor growth but is compensated by behavioral adaptation: only when the capacity for behavioral adaptation is exhausted do cachexia and hypophagia become overt. The effects of presence of tumor on the determinants of initiation and termination of meals are being examined in these respects.

(g) There has been intensive activity, in the form of advisory papers and memoranda, to a variety of Workshops and the the Advisory Committee in connection with the Diet, Nutrition and Cancer Program and in connection with membership of the Coordinating Committee of the Diet, Nutrition and Cancer Program. Organization and chairing of Workshop on Energy Metabolism in Cancer.

(h) Invited speaker:

1. American Institute of Nutrition, Wendell H. Griffith Memorial Symposium on Experimental Models for the Study of Obesity. Title: "The Hypothalamic Syndrome in Rats", April 1976.

2. First International Conference on Anorexia Nervosa. Title: "Extra-hypothalamic Controls of Feeding", October 1976.

3. U.S.-Japan Breast Cancer and Diet Epidemiology Meeting. Title: "Breakdown of Control of Feeding in Experimental Tumor Growth", March 1977.

4. American Society for Clinical Nutrition Symposium on Taste in Clinical Nutrition. Title: "Anorexia and Taste Disturbances in Neoplastic Disease", April 1977.

5. American College of Nutrition Symposium on Nutrition and Cancer. Title: "Anorexia and the Cancer Patient", June 1977.

Invited participant:

American Cancer Society Conference on Nutrition and Cancer Therapy, November 1976.

Invited special review member of Experimental Psychology Study Section, February 1977.

Significance to Biomedical Research and the Program of the Institute: The findings on control of feeding in tumor growth are throwing light on the nature of cancer cachexia and should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of Cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect ..... information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Proposed Course: Work on energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth. Objective 6, Approach 4.

Publications:

Morrison, S. D.: Theoretical Review. Control of food intake in cancer cachexia: A challenge and a tool. Physiol. Behav. 17: 705-714, 1976.

Morrison, S. D.: The hypothalamic syndrome in rats. Fed. Proc. 36: 139-142, 1977.

Morrison, S. D.: Extra-hypothalamic controls of feeding. In Vigersky, R. A. (Ed.), Anorexia Nervosa. Raven Press. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CBD 00942-18 LPP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effects of Gamma Irradiation on Nucleic Acids and Proteins: Interactions of Cancer Chemotherapy Agents and Radiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Riesz	Research Chemist	LPP NCI
Others:	S. Rustgi	Visiting Fellow	LPP NCI
	A. Joshi	Visiting Fellow	LPP NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of ionizing and ultra-violet radiation on nucleic acids and proteins and their constituents are being studied. The mechanism of the interaction of cancer chemotherapy agents with radiation is of interest, since such knowledge can be potentially useful in radiation therapy.

The present report includes electron spin-resonance studies of radicals produced by gamma-irradiation and ultra-violet photolysis of nucleic acids, proteins, their constituents and of chemotherapy agents of the intercalating type (e.g., actinomycin D, adriamycin).

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation react with DNA. For water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells, (b) halogenated pyrimidines which are incorporated into DNA and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells.

Studies of the mechanism of action of radio-sensitizers and radio-protectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

Methods Employed: Nucleic acids, proteins and their constituents were γ-irradiated either in the solid state at 2×10^{-5} torr or in aqueous solutions in an 800-curie Cobalt γ-source. Electron spin resonance studies were carried out with a Varian E-9 ESR Spectrometer connected to a Nicolet Lab 80 computer. For photolysis studies at specified wavelengths, a 1000-watt high pressure Mercury-Xenon arc source and monochromator were employed.

Major Findings:

1. ESR of spin-trapped radicals in gamma-irradiated aqueous solutions of nucleic acids and their constituents. (with A. Joshi, S. Rustgi) The gamma-radiolysis of deaerated neutral aqueous solutions of uracil, thymine, cytosine and of the corresponding nucleosides and nucleotides and of calf thymus DNA was investigated. For uracil and thymine the uv photolysis of aqueous solutions containing H_2O_2 was also studied. The short-lived radicals were spin-trapped by tert-nitrosobutane and identified by electron spin resonance spectroscopy. For all compounds two or more radicals were observed and these could be distinguished by following the thermal decay of the spin adducts. Radicals formed by the addition of H or OH at the C(5) or C(6) positions of the pyrimidine derivatives were observed in all cases.

Sodium formate was used as a scavenger for H and OH to identify the radicals formed by the hydrated electron.

Spin-trapped radicals in gamma-irradiated aqueous solutions of polynucleotides exhibited broad ESR lines. For DNA gel, additional narrow lines due to scission products were also found.

2. Reactions of hydroxyl radicals with N(1)-alkylated and dihydro derivatives of uracil, thymine and cytosine. (with S. Rustgi)
The H-addition radicals to the C-5 position of the 5.6 double bond of pyrimidines were made by H-abstraction from the C-6 position of the corresponding dihydro derivatives. These spin-trapped radicals had ESR spectra which could be clearly distinguished from the OH addition radicals characterized earlier. The additional hyperfine coupling due to a gamma-proton was observed. Methyl and ethyl derivatives of the dihydropyrimidines were also studied and H-abstraction from alkyl groups attached to N(1), C(6) and C(5) positions was observed.

The effects of N(1) substitution on the N(1) nitrogen and C(6) proton hyperfine couplings were measured. As the substituent group changed from H to methyl to sugar, the N(1) coupling decreased, the C(6) proton coupling increased and the hyperfine coupling due to the C(5) proton became unresolved. These observations help to explain the loss of detailed hyperfine structure in nucleosides and nucleotides.

3. ESR study of spin-trapped radicals formed during photolysis of aqueous solutions of amides and H_2O_2. (with S. Rustgi)
Free radicals formed by the reactions of OH radicals with amides and their N-methylated derivatives in aqueous solutions have been studied. The OH radicals were produced by UV photolysis of H_2O_2 and the short-lived amide radicals were converted to more stable nitroxide radicals by addition to a spin-trap, tert-nitrosobutane. The spin-trapped radicals were identified by ESR spectroscopy. For acetamide, chloroacetamide, malonamide, succinamide and propionamide, the observed radicals were formed by H-abstraction from the carbon attached to the carbonyl group. The H atom attached to the carbonyl group was abstracted in formamide. For N-methyl acetamide, N,N-dimethyl acetamide and the corresponding formamide derivatives H abstraction occurred only from the N-methyl groups. The establishment of the site of OH attack is of considerable significance in the identification of radicals from peptides. Studies of the reactions of OH radicals with dipeptides also show that alpha-carbon radicals are only found on the carboxyl terminal residue. The nonequivalency of the amide protons was observed in the spin-trapped radicals for acetamide, formamide, malonamide, succinamide and propionamide.

4. ESR of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydroxyl radical. (with A. Joshi, S. Rustgi)
The radicals produced by reactions of hydroxyl radicals with amino acids in aqueous solutions have been investigated. Hydroxyl radicals were formed by uv-photolysis of hydrogen peroxide and the short-lived amino acid radicals were spin-trapped by tert-nitrosobutane and identified by electron spin

resonance spectroscopy. Nineteen amino acids were studies, and several radicals were identified which have not been observed previously by other methods. Only side-chain radicals were identified for alanine, threonine, aspartic acid, asparagine, lysine, phenylalanine, tyrosine, proline and hydroxyproline; whereas for glycine the C(2) carbon radicals and side-chain radicals were assigned to valine, leucine, isoleucine, serine, glutamic acid, glutamine, arginine and methionine.

5. ESR of spin-trapped radicals in aqueous solutions of amino acids. Reactions of the hydrated electron. (with S. Rustgi, A. Joshi, and F. Friedberg, Department of Biochemistry, Howard University Medical School) Hydrated electrons were generated by gamma-radiolysis of deaerated aqueous solutions containing a sufficient concentration of sodium formate or tert, butanol to scavenge 95% of the hydroxyl radicals. Twenty of the common amino acids were investigated and radicals formed by reductive deamination were spin-trapped and identified in all cases except tyrosine. For phenylalamine and imidazole, radicals produced by electron addition followed by proton transfer were observed. For histidine and arginine, no evidence for the reaction of the hydrated electron with the side chain was observed. However, the side chain of methionine reacts with the hydrated electron to break the carbon-sulfur bond on either side of the sulfur.

The ESR spectra from spin-trapped deamination radicals are very characteristic except for glycine they consist of a 3.40 to 5.70 doublet due to the β-proton at the C(2) position. This secondary doublet is further split into a small doublet or a 1:2:1 triplet or a 1:3:3:1 quartet due to one, two or three equivalent gamma-protons at the C(3) position. In the case of glycine the secondary structure consists of a large 1:2:1 triplet (8.45G) due to two equivalent β-protons.

6. ESR of spin-trapped radicals in aqueous solutions of dipeptides. Reactions of the hydroxyl radical. (with A. Joshi, S. Rustgi, and H. Moss) The radicals formed by the reactions of hydroxyl radicals with dipeptides in their zwitterionic forms were studied at room temperature. The hydroxyl radicals were generated by uv-photolysis of hydrogen peroxide and the dipeptide radicals were spin-trapped with tert-nitrosobutane and identified by ESR. Most of the experiments were carried out in D_2O solutions. Some of the dipeptides with structures glycyl-X, X-glycine, X-X and X-Y, where X or Y = Gly, Ala, Val, Leu, Ile, Ser, Thr, Asp, Glu, Lys, Arg, Met, His, Phe, Tyr, Pro or 4-Hyp were investigated. For peptides of the type X-Gly and X-Ala, the radical formed by abstraction from the carbon located between the peptide nitrogen and carboxylic group was observed along with the side chain radicals. For X-Y dipeptides where Y is not glycine or alanine, only side chain radicals were identified in most cases. The spectra observed from $(Gly)_n$ and $(Ala)_n$ peptides (n-3,4 or 5) were similar to but less resolved than the spectra for the corresponding dipeptides. Glycylglycine was also studied over the pD range from neutral to 1.4 and the pK value of the carboxyl group of the spin-adduct was found to be ~2.

In acid solutions (pH2-3), the ESR signal due to di-tert-butyl nitroxide could be photobleached, thus permitting the identification of additional backbone radicals in formyl and acetyl derivatives of phenylalanine, lysine and methionine.

It seems likely that the present approach can be employed with much larger peptides. For ribonuclease-S-peptide, containing 20 amino acid residues, radicals on alanine and serine residues could be identified.

7. Modification of Radiation Damage in DNA by Cancer Chemotherapy Agents. (with A. Joshi)
Certain cancer chemotherapy agents of the intercalcating type (e.g., actinomycin D, adriamycin, daunomycin) potentiate the effects of ionizing radiation.

In order to understand this interaction, some of these drugs were spin-trapped with the future aim of studying the orientation of the intercalators with respect to the DNA bases. This could be done by investigating the ESR spectra of partially oriented samples of spin-labeled intercalator-DNA complexes as a function of orientation in the fixed magnetic field.

Spin-adducts were made by generating OH radicals by the photolysis of aqueous solutions containing hydrogen peroxide, the intercalator and t-nitrosobutane. The ESR spectra of the spin-adducts obtained from actinomycin-D, actinomin and from 2-aminophenoxazone-3 were very similar and indicated H-abstraction from a methyl group.

Spin-adducts have also been obtained from daunorubicin and adriamycin and from some compounds which are not chemotherapy agents, such as chloroquin and 10-acetyl phenothiazine. The effects of the chemotherapy agents on the yield and nature of the radicals produced in DNA by gamma-irradiation will be investigated.

Significance to Biomedical Research and the Program of the Institute: Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained. Objective 6, Approach 1.

Proposed Course: To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be investigated.

Publications:

Kominami, S. and Riesz, P.: ESR Studies of gamma-irradiated wet DNA. Radiat. Res. 67: 173-177, 1976.

Kominami, S., Wee, V. T., and Riesz, P.: ESR Study of the effect of visible light on gamma-irradiated DNA and its constituents at 77° K. Radiat. Res. 69: 213-222, 1977.

Joshi, A., Rustgi, S., and Riesz, P.: ESR of spin-trapped radicals in gamma-irradiated aqueous solutions of nucleic acids and their constituents. Int. J. Radiat. Biol. 30: 151-170, 1976.

Rustgi, S., Joshi, A., Moss, H., and Riesz, R.: ESR of spin-trapped radicals in aqueous solutions of amino acids: Reactions of the hydroxyl radical I. Int. J. Radiat. Biol. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00941-21 LPP

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunogenetic Factors Affecting Bone Marrow Transplantation in Irradiated Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. E. Uphoff Research Biologist LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Radiation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bone marrow transplantation in lethally irradiated mice was used to investigate immunogenetic factors affecting the severity of the graft-versus-host reaction. A high leukemic strain of mice was treated with lethal irradiation and inoculated with compatible marrow to study incidence, latency and genotype of the leukemias. Leukemia induction in donor marrow occurred earlier when the marrow was most compatible with the recipient. Two methods of altering the incidence of leukemia were investigated. The most effective method apparently alters the environment while the other apparently alters the susceptibility of the transplanted cells to malignant transformation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To facilitate bone marrow transplantation as an adjunct to administration of toxic levels of irradiation or chemotherapeutic agents used in treating cancer. Bone marrow transplantation is also useful for treating combined immune deficiency diseases, aplastic anemia and accidental over exposure to irradiation and toxic chemicals.

Methods Employed: Multiple inbred strains and subtrains of mice representing the major histocompatibility genotype (H-2), reciprocal F_1 hybrids homozygous and heterogygous for their H-2 alleles, and specially derived substrains are bred and maintained as a closed colony for these experiments since the health of the animals before and during experimentation is of paramount importance for accurate interpretation of survival data. The nature of the breeding program requires that data from multiple small experiments be accumulated over relatively long periods of time before meaningful patterns emerge. Mice are exposed to lethal total body X irradiation and engrafted with marrow cells from different donors to test effects of genetic disparity, sex, and parity status on engraftment and severity of the graft-versus-host reaction (GVHR). The ultimate goal is to devise methods of preventing the GVHR or controlling the lethal effects of a strong reaction while utilizing possible beneficial effects of less severe reactions for their immunotherapeutic effects against cancer cells.

Major Findings: The modified Westinghouse 250KV X ray machine with dual opposing tubes used for irradiating experimental animals since 1948 was replaced in April 1976 by a similarly modified Philips machine. Using the same filtration the rate of output of the new equipment was 126R/minute as compared with the 139R/minute previously administered. All other parameters were adjusted to produce a calibrated dose equivalent to that previously used. However, when tested in the biological system a qualitative difference in the effect of the irradiation produced by these two machines was detected, e.g., the slope of the dose response curves for several inbred strains was steeper with the Westinghouse machine than with the Philips machine. In addition, some bone marrow transplantation experiments that showed remarkable reproducibility over many years were no longer reproducible with the new equipment. On one previous occasion between 6/28/72 and 8/30/72, there was a failure to reproduce certain experimental data under conditions that had previously been very reliable. During this 8 week period the auxillary line voltage regulator was in need of repair and had been bipassed. The expected results were obtained when the line voltage regulator was reinstalled. This affect of the line voltage regulation was confirmed by running dual experiments on the same day with and without the line voltage regulator. The new Phillips equipment was not installed with the additional line voltage regulation. The comparison between data obtained in July and August 1972 was sufficiently consistent with that obtained after April 1976 to warrant the installation of additional line voltage regulation on the new equipment which is scheduled for completion in May 1977. The only way of detecting the qualitative difference in

output between the two machines has been with this biological system which may be sensitive enough to reflect biological effects equivalent to an estimated 25R difference in exposure.

One of the ongoing experiments on which data is being accumulated is the treatment of high leukemic AKR mice with lethal irradiation and marrow replacement in an effort to control the course of this disease. Few mice survive longer than a year without developing leukemia, 85% of which is of thymic origin and 10% of non-thymic origin. AKR mice 3-11 months of age were exposed to 800R and inoculated intraveniously with compatible ($H\text{-}2^K$) marrow from C58, C3HfB, CBA and ST donors respectively. Mice that were grossly leukemic at the time of treatment died during treatment or shortly thereafter as a result of the destruction of a relatively large volume of tumor tissue. Of the group that survived treatment there was frequently a delay in the onset of the disease resulting in a prolongation of the expected life span of 1 year. Leukemias arising in these chimeras were transplanted into AKR and mice of the marrow donor strain to determine the genotype of the neoplasm. Tumors arising between 30 and 60 days after treatment were primarily of AKR tissue origin indicating that preleukemic cells were not destroyed by this dose of irradiation or by the mild graft-versus-host reaction. During the second to fourth month after treatment, tumors were of either donor or recipient genotypes. After the fourth month, a single AKR tumor occurred during the 8th month. This single case probably represented the loss of the marrow graft with reversal of the chimeric state since more neoplasms of donor tissue origin occurred after 60 days than those of AKR origin. The ratio of donor/recipient tumors was a function of the marrow donor strain: - C58(10/2), C3HfB(15/15), CBA(20/14) and ST(66/21). Three mixed tumors specific for both donor and recipient genotype, occurred 2.5 - 4.5 months after they were grafted with ST marrow. Leukemias of donor tissue origin occurred earlier among recipients of the most compatible ST marrow than among recipients of the least compatible C58 marrow. It is clear that without altering either the environment or the susceptibility of the transplanted cells to malignant transformation, leukemia may be induced in the marrow grafts of AKR recipients. The induction of leukemia in donor cells has been reported for clinical marrow transplantation.

Two methods of reducing the incidence of leukemia in AKR mice have been found. They appear to involve different mechanisms. Both methods involve the use of pretreated AKR marrow. Lethally irradiated AKR mice inoculated with AKR or CBA marrow from normal young donors have a 65% incidence of leukemia. When these two marrows are mixed and incubated for 30 minutes at 26°C before inoculation into the lethally irradiated AKR mice the incidence of leukemia is reduced to 5%. Washed CBA erythrocytes (rbc) are equally effective antigens for the incubation. However (AKR x CBA)F_1 or (CBA x AKR)F_1 hybrid rbc are less effective (39%, 23% as compared with 5% for CBA rbc).[1] The effectiveness of this treatment is time and temperature dependent and is affected by the antigenicity or immunogenicity of the antigen used in the incubation. Since hybrid marrow incubated with parental strain antigens is ineffective in reducing the incidence of leukemia, coupled with the fact

that the thymus glands of these mice is greatly reduced, it is proposed that there is specific immunogenetic effect directed against the thymus as the target organ. Since the effectiveness of this treatment depends upon the recognition of a specific level of foreignness to achieve the best results the control of leukemogenesis in these experiments would appear to depend upon an altered environment.

The second method of reducing the incidence of leukemia is less effective but may represent an alteration in the susceptability of the inoculated marrow to malignant transformation. In this case lethally irradiated AKR mice were inoculated with AKR marrow preincubated with bovine serum albumin (BSA) for 30 minutes at 26°C. The incidence of leukemia was 21% if recipients were under 14 weeks of age but 78% if recipients were 17-19 weeks of age. The success of this treatment requires that both recipients and donors must be young animals for if marrow from donors 17-19 weeks of age is pretreated and inoculated into young recipients the incidence of leukemia is already 60% with 80% of the mice dead. An irreversible change occurred between 14-17 weeks of age after which this treatment no longer interrupts the malignant process. In an attempt to determine whether the transformation occurred in donor or recipient cells CBA marrow was incubated with 10% BSA and inoculated into young AKR recipients. There was a greatly reduced 60 day survival to 40%. A sex difference in the incidence of leukemia is emerging with less than 50% of the mice dead the incidence in males is 28% compared to 70% for females. Ten out of the 11 leukemias have been transplanted to AKR and CBA recipients. Four tumors have grown in CBA mice and 1 in AKR mice, 1 failed to grow and 3 tests are incomplete. Before it was realized that all experiments would require confirmation when the line voltage regulator is installed, AKR mice were treated with AKR marrow preincubated with 1% BSA to determine if the concentration of antigen was a critical factor and 10% dextran was substituted for BSA to test the effect of a second soluble antigen. Leukamias have not as yet occurred in either of these later groups.

Significance to Biomedical Research and the Program of the Institute: Clinical marrow transplantation is being used in various Institutes throughout the world. Among the problems encountered in addition to the GVHR has been the induction of leukemia in the transplanted donor marrow. Although one hesitates to extrapolate from mouse to man, the mouse model for investigating the immunogenetic factors involved in marrow transplantation is the only one available. Time has demonstrated that the mouse models used in this research program have accurately forecast problem areas for clinical transplantation. Leukemias of donor genotype occurred in AKR and other chimeras and were first reported 20 years age. The relationship between donor compatibility and the "remission time" is a recent contribution. Although immunologists have long considered erythrocytes an extremely poor source of tissue antigens, rbc have been successfully used for their antigenic properties in controlling both the severity of the GVHR and the incidence of recurrent leukemia and for the induction of tolerance in the mouse model.

It was recently found that patients given transfusions prior to receiving kidney grafts have significantly longer graft survival than those without transfusion - another paradox contrary to the dogma perpetuated by immunologists. There should, no doubt, be a reevaluation of this "poor" antigen for its usefulness in other systems. The use of sex chromosome markers to demonstrate successful clinical marrow transplantation, a practice of long standing, was recently demonstrated to produce significantly poorer long term survival than cases that were matched for sex. Again there is a close correlation between the clinical experience and that previously reported in mouse models investigating the GVH reaction against sex linked antigens.

Proposed Course: The time and animals required to test the new X ray equipment has greatly hampered progress during the past year. Until the X ray equipment is further modified and appropriate tests conducted, none of the ongoing experiments can be completed. The installation of a switching mechanism to bipass the voltage regulator will enable the investigation of the effects of the line voltage regulator on this biological system. In the interim, base-line information is being accumulated for this purpose. Other aspects of this project reported in previous years will be continued when it is satisfactorily demonstrated that the dose of irradiation used produces the same biological effects as those produced by the Westinghouse equipment.

Publications:

Uphoff, D. E.: Mechanisms of graft-versus-host reaction: An immunogenetic appraisal. Transplantation Proc. 8: 515-519, 1976.

Uphoff, D. E.: Runt disease in suckling hybrid mice: Substrain differences and litter seriation. Transplantation Proc. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00936-07 LPP

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Repair of Sublethal Radiation Injury in Mice.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. W. Smith Research Physiologist LPP, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Radiation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Summary: In mice given sublethal whole-body irradiation it appears that persisting hematopoietic rather than intestinal damage is responsible for the reduction in ability to tolerate a second irradiation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To understand the physiological basis of persisting sublethal radiation damage, particularly in regard to effects of subsequent irradiation on survival.

Major Findings: In mice given an initial sublethal irradiation to the whole body there is generally a persisting reduction in ability to tolerate a subsequent irradiation. In our experiments the effect of this initial dose was a reduction of 16% in the LD_{50} determined two to six weeks later, corresponding closely to the early observations of Blair and others. When the marrow was protected by leg shielding and the inital dose almost doubled, the subsequent challenge showed the same LD_{50} as leg-shielded controls given no prior irradiation. It thus appears that the persisting irrepairable damage is of hematopoietic origin and not related to the gut.

Significance for Cancer Research: Since radiation therapy for cancer involves repeated exposures, knowledge of the duration and persisting effects of sublethal injury are of primary importance.

Proposed Course of Project: None, because of retirement.

LABORATORY OF THEORETICAL BIOLOGY

SUMMARY

July 1, 1976 through September 30, 1977

Summary Report

The activities of the Laboratory of Theoretical Biology (LTB) fall into several broad areas: membrane biophysics, immunology, carcinogenesis, macromolecular configurations, kinetics of metabolic systems, computational methodology, and theory related to modeling. Most of the work is theoretical, but experiments are also carried out in the laboratory. Much of the theoretical work and modeling is done in collaboration with experimental groups at NIH and elsewhere.

The work on membranes includes several areas of interest: lipid bilayer systems, vesicle-cell fusion, mechanisms of cell lysis, and diffusion of cell surface receptors in membranes (R. Blumenthal, J. Weinstein, P. Dragsten). This work is both experimental and theoretical.

The interaction between lipid vesicles and cell membranes is of interest because of its close analogy with events in such physiological processes as secretion and viral infection, and also because lipid vesicles are currently under intensive study as vehicles for the delivery of physiologically-active agents to cells or tissues. Our studies of vesicle-cell interaction are done with vesicles containing varying concentrations of the fluorescent dye 6-carboxyfluorescein. At high concentration the fluorescence is self-quenched. As the vesicle contents are diluted - through release into cytoplasm or medium - fluorescence appears. By observing the fluorescence through a microscope and by quantitating its intensity using fluorometry and flow microfluorometry, it is possible to study the binding and fusion of vesicles with cells, and to define the variables controlling these processes.

Major findings in this work, done with human lymphocytes, are as follows: (1) Vesicle-cell binding can be achieved or eliminated by manipulation of the vesicle lipid composition. (2) There are saturable "sites" for vesicle-cell fusion and also a non-saturable component of the process. (3) The inhibitors of metabolism investigated were found to affect primarily the efflux of liposome-encapsulated material from the cells. (4) Antibody can be used to "target" antigen-coated vesicles for antigen-coated cells, but the consequent vesicle-cell binding does not lead to incorporation of vesicle contents into the cells. This last finding suggests one possible difficulty in the use of targeted lipid vesicles as delivery systems.

Complement lysis: (1) Vesicles containing 6-CF provide a sensitive assay for complement lysis, permitting detailed study of the kinetics of the process. (2) Both small unilamellar and large multilamellar vesicles containing DNP are lysed by the combination of anti-TNP antibody and complement. (3) The rate of lysis is ten to twenty fold enhanced by addition of cholesterol to the vesicle forming solution (DOL:cholesterol=1.5:1).

The mobility in bimolecular lipid membranes (BLM) of dextrans derivatized with controlled amounts of fatty acid, rhodamine, and TNP has been studied using photobleaching techniques. Diffusion coefficients of these substances were measured, and patching was demonstrated upon addition of anti-TNP or con A. These studies are part of an effort to elucidate the roles in membrane function played by membrane fluidity and the diffusion of cell surface receptors.

In the area of theoretical immunology (C. DeLisi, A. Thakur) analysis of plaque assay continued with particular emphasis on the experimental conditions which must be fulfilled for inhibition experiments to yield thermodynamic information. Work on a related problem was also initiated, viz, obtaining from equilibrium binding data, the distribution of equilibrium constants characterizing the interaction of a heterogeneous receptor population with homogeneous ligand. The problem was approached by a method of moments.

Mathematical models have been developed for describing the dynamical events which occur on the lymphocyte plasma membrane subsequent to interaction with antigen. The models have been simulated numerically in an attempt to understand whether these events are important in the control of the quality of antibody (i.e., affinity) produced during a response, and if so, how they exert their influence.

Work was also initiated on mathematical modeling of carcinogenesis. The work in this area attempts to formulate a broad conceptual framework within which one can incorporate as much of the phenomonology of carcinogenesis as possible, and to develop mathematical models for specific aspects of carcinogenesis within this framework, that lead to testable predictions or suggestions for original experiments. Efforts are currently directed toward both fundamental and applied theory. The former category presently includes models of the host defense interacting with the primary tumor, and models of metastasis formation. In the latter category a specific model for breast cancer epidemiology has been proposed.

Studies on the kinetics of protein folding and the denaturation and renaturation of macromolecules are being continued in an effort to understand the basic mechanisms (R. Jernigan, S. Szu). A previous model, appropriate for polypeptides has been extended to treat conformational relaxation in DNA. Renaturation of DNA requires a more complex treatment because of the effects of a bimolecular reaction step for the formation of initial helix units. Good agreement was found with DNA denaturation experiments, but not all renaturation data can be explained with the present theory.

A new method has been developed to predict probable protein secondary conformations from their sequences. Interatomic interaction energies are calculated in order to obtain probabilities of various secondary structure regions. A selection process based on local equilibrium yields good predictions of α helix and β strand regions compared to known crystal structures; whereas the alternative scheme of minimizing the total molecular secondary structure energy is not as successful. These results yield relative stabilities of the various regions which may represent the conformations of partially denatured proteins.

The modeling of endocrine and other metabolic and dynamic systems has continued (M. Berman). These include lipoproteins (L. Zech, D. Foster), glucose (E.A. McGuire, D. Foster), insulin (E.A. McGuire), thyroid hormones (R. McGuire), adenylate cyclase (L. Zech), receptors (A. Babcock). In the lipoprotein area, the model developed over the years for the synthesis, catabolism and interconversion of very low density, low density and high density lipoproteins is being confirmed and extended through new data and is widely used for the study of abnormal metabolism (collaborations with Drs. E. Schaeffer, B. Brewer and R. Levy, NHLI and Dr. C. Malmendier, Bruxelles and Drs. S. Grundy and D. Steinberg, La Jolla). Our analyses suggest that lipoprotein particle size plays an important role in the metabolic pathways, and can greatly influence the interpretation of kinetic data. Drugs, such as atromid, can reduce very low density lipoprotein production through a shift to the production of smaller (intermediate and low density) particles - not necessarily affecting the cholesterol carrying capacity in plasma. We have also shown heterogeneity in the LDL particles and have further validated the existence of a slow turning over VLDL compartment in type III patients (Dr. W. Hazzard, Seattle) and type IV patients (Dr. W. Fisher, Univ. of FL). The kinetic studies of the cholesterol moiety is also continuing with the VA group in Richmond (Drs. L. Swell and C. Schwartz) and a model for bile acid and bile cholesterol has been formulated. The emerging general model for lipoprotein kinetics suggests new ways of looking at abnormalities in lipid metabolism. As an adjunct to this, the delipidation process of chilomicron triglycerides in vitro was modeled and the control of this by liberated free fatty acids and the presence of albumin as a receptor was formulated and quantified.

In studying the glucose-insulin control system, the major effort went into the insulin subsystem. An analysis of data in normal, obese, aged and diabetic populations in man show relatively comparable kinetics although reduction in the ability of tissues to retain insulin is suggested in diabetes and obesity (collaboration with Drs. R. Andres and J. Tobin, NIA). New facets of insulin kinetics involving further compartmentalizations have been shown to exist in a series of experiments in rabbits using high and low affinity insulins (collaboration with Drs. Zeleznik and Roth, NIAMDD). Heterogeneities of the injected insulins have also been shown from the kinetics analysis. Special experiments to follow-up on these are now in progress.

The interconversion of gluconeogenic precussors (lactate, alanine) and their incorporation into glucose in the dog have been quantified to permit a more complete accounting of the glucose carbon sources (collaboration with Dr. G. Heteney, Univ. of Ottawa). Changes in these pathways produced by insulin deficiency are being investigated. These studies are part of our effort to develop a comprehensive model of the glucose-insulin system.

A number of kinetic models have been developed for various systems in collaboration with other investigators or as adjuncts to ongoing work. These include: 1) The synthesis and metabolism of thyroxine binding globulins (TBG) and the effects of estrogen, hyper- and hypothyroidism on these (with Drs. Robbins and Daniels, NIAMDD). 2) The synthesis and kinetics of thyroglobulin (with Drs. Rall and Burkhardt, NIAMDD). 3) The binding and cyclic AMP activation of prostaglandin in normal and transformed cells (with Drs. Pastan and Davies, NCI). A high degree of positive cooperativity was demonstrated for

the activation process. 4) The study of LDL and HDL interactions with receptors and their internalization in fiberblasts. 5) The study of zinc metabolism in man and its absorption by the gut.

The development of methodology and formalisms for dealing with large metabolic systems has continued (A. Rescigno). The theory for kinetics of chemical reactions using a stoichiometric approach had been further developed and algorithms for their solution improved (D. Park). The SAAM computer program (M. Berman and M. Weiss) developed for the modeling of complex, non-linear systems has also been further improved by extending its power and versatility. Conversational SAAM was extended and is more widely used. A two day workshop on SAAM was conducted by LTB in Oak Ridge, Tennessee and was attended by about 30 SAAM users.

The Laboratory of Theoretical Biology also consults with investigators on various problems and assists in the use of the SAAM program. It also supports a PDP-11/20 data processing system for which special and general programs have been developed for data processing and plotting (F. Millar).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08300-05 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

SAAM Computer Program

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	M. F. Weiss	Mathematician	LTB NCI
	R. McGuire, Ph.D.	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Theoretical Biology

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INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing development of a computer system (SAAM) for the simulation, analysis and modeling of bio-kinetic systems. Further development of a conversational mode of operation, increased versatility of applications and automation are planned.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a general purpose computer program for modeling biokinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. Initiated in 1959, the program continues to be expanded and revised as new features are added.

Major Findings: The computational power of SAAM was further expanded. Improvements include a faster algorithm for equilibrium kinetics, extension of the Forcing Function and input function capability. New commands were also incorporated into conversational SAAM making it easier to use. A workshop for SAAM users was given in Oak Ridge by this group and material on modeling, compartmental systems and SAAM was developed. A new manual for SAAM27 was also put together.

Significance to Biomedical Research and the Program of the Institute: The SAAM computer program has been a most essential tool in the modeling of various systems described elsewhere in this Laboratory report. Its wide use in other centers in the United States and elsewhere is further evidence of its value in biomedical research.

Publications:

Authors: Berman, M., Weiss, M. F.: SAAM Manual. Washington, D.C., US Govt. Printing Office, DHEW Publication No. (NIH) 76-730, 1977, 175pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08301-05 LTB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modeling Theory and Applications

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	D. Foster, Ph.D.	Staff Fellow	LTB NCI
	A. Babcock, Ph.D.	Staff Fellow	LTB NCI
	R. McGuire, Ph.D.	Guess Worker	LTB NCI

COOPERATING UNITS (if any)

J. E. Rall, NIAMDD: J. Robbins, NIAMDD; I. Pastan, NCI; M. Rodbell, NIAMDD

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SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of various kinetic and metabolic systems by the use of mathematical models carried out in collaboration with other investigators. These include binding, internalization and activation of receptor systems (insulin, low density lipoprotein, prostaglandin, glucagon, zinc metabolism in man, amino acid transport, thyroglobulin synthesis and thyroxine binding globulin metabolism. Specifically, these studies also serve to test and develop the SAAM computer program and modeling theory.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Analysis of data on metabolic systems, and further development of modeling theory and techniques through the applications of the SAAM computer program.

Major Findings: The binding and activation of Prostoglandin E_1 (PgE_1) to membranes of clones of normal rat kidney fibroblasts (NRK) has been modeled. (Zech). The model results suggest that there is a high order of positive cooperativity in the activation process and a negative cooperativity in binding. These findings also hold for some transformed cell lines (collaboration with Drs. P. Davis and I. Pastan).

A model has been developed for TBG metabolism and the effect on this by estrogen and hypo- and hyperthyroidism. The model suggests an acute effect on the synthesis of TBG by estrogen as well adaptive longer range effects (collaboration with Drs. Glinoer and Robbins).

The synthesis of thyroglobulin in vivo has been studied in an effort to determine the sequence of formation of proteins A, B and C. Unfortunately, despite the careful data, this could not be fully resolved. It was found, however, that a large portion of newly synthesized thyroglobulin is hydrolyzed before it diffuses into the colloid. The model also suggests that there is some conversion of protein B to C (collaboration with Drs. A. Brunkhardt and J. E. Rall).

A model for zinc metabolism in man is being constructed (D. Foster). At present the data base is limited to five days. Over this period of time the model fits data from both i.v. and oral administrations. The rate of zinc absorption through the gut has also been derived through the use of the model.

The work on receptors and amino acid transport (A. Babcock) has not yet reached a state in which major findings can be reported.

Significance to Biomedical Research and the Program of the Institute: The methodology of modeling is most essential for studying the behavior of complex systems for both normal and abnormal states.

Proposed Course: This is a continuing process for the development of theory for and the application of modeling techniques.

Publications:

Berman, M.: Kinetic models for absorbed dose calculations. NM/MIRD. New York, Society of Nuclear Medicine, Inc., 1977.

Glinoer, D., McGuire, R. A., Gershengorn, M. C., Robbins, J. and Berman, M.: Effects of estrogen on thyroxine-binding globulin metabolism in rhesus monkeys. Endocrinology 100: 9-17, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08303-05 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Lipid Model Membrane Studies of Ion Transport Membrane Fusion and Cell Surface Mobility

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.P. Blumenthal, Ph.D.	Visiting Scientist	LTB NCI
	J.N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI
OTHER:	P. Dragsten, Ph.D.	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)
P. Henkart, Ph.D., S.O. Sharrow, J. Wunderlich, M.D. IB, NCI, NIH
D.E. Wolf, Ph.D., J. Schlessinger, Ph.D., E.L. Elson, Ph.D., and W.W. Webb, Ph.D.
Dept. of Chemistry, and School of Applied Physics, Cornell University

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipid bilayer membranes are used to study various aspects of membrane structure and function. Electrical properties of bimolecular lipid membranes (BLM's) are measured to study: (1) mechanisms of opening and closing ion channels; (2) properties of ionophores isolated from natural cell membranes; (3) mechanisms of immune cytotoxicity; (4) relationship between ion permeability and mitogenesis. Diffusion and patching of fluorescent surface receptors in BLM's is studied by the technique of fluorescence photobleaching recovery: a small spot on the fluorescent-labeled BLM surface is photolytically bleached by brief exposure to an intense laser beam, and subsequent recovery of the fluorescence is monitored. Lipid bilayer vesicles containing a high concentration of the fluorescent dye 6-carboxyfluorescein (6-CF) are used to study: (1) mechanisms of liposome-cell interaction (e.g., surface adsorption, fusion or endocytosis), and (2) the mechanism of lysis of the vesicles by drugs or by complement. The vesicles are incubated with human peripheral blood lymphocytes, and the resulting fluorescence is observed by microscopy, by fluorometry and by use of the fluorescence-activated cell sorter.

Project Description:

Objectives: To study the physical mechanisms of ion transport in reconstituted membranes. To develop the lipid bilayer membrane as an assay for ionophoric materials isolated from natural cell membranes. To study the interaction of lymphocytes with lipid bilayer membranes as a model for lymphocyte-mediated lysis of target cells. To study the role played in cell membranes by the mobility and distribution of cell surface antigens. To develop fluorescent techniques for studying interaction of lipid bilayer vesicles with lymphocytes, with the possibility of targeting drug-containing vesicles for specific cells. To develop fluorescent techniques for studying lysis of lipid vesicles by drugs and by complement. To study the relationship between ionophoric and mitogenic properties of lymphocyte-activating compounds.

Methods Employed: The bilayer membranes are formed from natural membrane extracts, oxidized cholesterol, or pure lipids in an aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. In the fluorescence photobleaching experiments a small spot on the fluorescence-labeled cell surface is photolytically bleached by brief exposure to an intense laser beam, and subsequent recovery of fluorescence is monitored with an attenuated laser beam.

Single-bilayer vesicles containing 200 mM 6-carboxyfluorescein are formed by sonication from dioleoyl phosphatidyl choline (DOL) or distearoyl phosphatidyl choline (DSL), DOL + cholesterol, or DOL + DNP-caproyl-phosphatidylethanolamine (DNP-cap-PE). Free dye is removed on a Sephadex G-50 column. At the high concentrations (100mM-200mM) fluorescence of 6-CF is self-quenched. Relief of self-quenching when the dye is diluted from vesicles into the medium is a measure of leakage of dye from vesicles (e.g. by lysis induced by the complement system). In the complement studies purified rabbit IgG and quinea pig complement are added to the vesicle suspension and the time course of fluorescence increase (i.e. leakage) is measured. In the fusion studies the vesicles are incubated with human peripheral blood lymphocytes, and the resulting fluorescence is observed by microscopy, by fluorometry, and by use of the fluorescence-activated cell sorter. In the targeting studies vesicles made with DNP-cap-PE are incubated with TNP-modified human lymphocytes and anti-TNP antibody.

Major Findings:

Fusion Studies: (1) Lymphocytes, macrophages and chinese hamster cells incorporate dye from vesicles (2) Fluorescence microscopy indicates that the dye is released into the cytoplasmic compartment, with less fluorescence in the nucleus; there are also local accretions of dye whose interpretation is uncertain (3) The fluorescence efficiency of the dye in the cell is 30% less than the fluorescence of 6-CF in medium buffered at pH=7.4; the main factor in reducing the efficiency is the lower pH in the cell (probably 6.8); (4) Release of material within the cell is distinguished from contamination with intact vesicles; the intact vesicles are bound to the cell surface. (5) Frequency

histograms of cells measured in the fluorescence activated cell sorter show homogeneous population of dye-containing lymphocytes. (6) Altering the vesicle lipids with respect to charge and fluidity does not alter the amount of fusion; in the case of solid vesicles made of distearoyl lecithin (phase transition temperature 56°C) there is a large amount of vesicle binding not seen with fluid vesicles made up DOL, but the same amount of fusion. Incorporation of the charged phospholipid phosphatidyl serine (PS) (PS:DOL=1:10) in the presence or absence of Ca^{2+} did not affect fusion or binding. (7) Fusion of DOL, DSL, DOL + PS vesicles was inhibited by 20-30% in the presence of sodium azide (0.1%) and 2-deoxy-D-glucose (50mM) in combination, Ca^{2+} did not effect the inhibition. (8) Study of the time course of incorporation of dye from vesicles and of incorporation of free dye showed that cell fluorescence levelled off at about one hour determined by a balance of influx and efflux of the dye. (9) Inclusion of sodium azide and 2-deoxy-D-glucose inhibited one major component of the efflux and the inhibitor appeared to enhance the uptake of dye at longer incubation times. (10) Careful dose-response curves of initial rates of vesicle-mediated transfer as a function of vesicle concentration revealed saturable sites and nonsaturable components of fusion. (11) Cold vesicles (i.e not containing 6-CF) blocked the saturable sites. Fetal calf serum and bovine serum albumin also inhibited vesicle mediated transfer but bovine IgG had no effect. (12) Proleolytic digestion of the lymphocyte surface had no effect on the vesicle-mediated transfer. (13) When vesicles made with antigenic lipid (DNP-cap-PE) where incubatedwith TNP-modified human lymphocytes and anti-TNP antibody the bivalent antibody acted as a bridge binding 5,000-15,000 haptenated vesicles to each haptenated cell. (14) Targeted binding led to little or no increase over spontaneous background levels of fusion.

Photobleaching studies: (1) Derivatives of dextran to which controlled amounts of fatty acid, rhodamine, and TNP have been covalently bound can be incorporated into BLM's from the aqueous medium. (2) The membrane bound "antigens" were distributed homogeneously and had a diffusion coefficient of $D=2.1\ 10^{-8}$ cm^2/sec on oxidized cholesterol and $4.5\ 10^{-8}$ cm2/sec on egg phosphatidyl choline bilayers. (3) Addition of anti-TNP or con A reduced the diffusion coefficient of the "antigen" and resulted in patching whereas the diffusion coefficient of a lipid probe was unaffected. (4) Sodium azide, colchicine and cytochalasin B did not affect the motion of these molecules on the bilayer.

Complement lysis: (1) Vesicles containing 6-CF provide a sensitive assay for complement lysis whose kinetics can be studied in detail. (2) Both small single-lamellar vesicles and large multilamellar vesicles are lysed by the TNP-anti DNP-complement system. (3) The rate of lysis is ten to twenty fold enhanced by addition of cholesterol to the vesicle forming solution (DOL: cholesterol=1.5:1).

Significance for Biomedical Research and the Program of the Institute: Increasingly it appears that ion permeabilities play an important regulatory role in cell maintenance and division; the lipid bilayer has been proven a sensitive tool for study of the molecular properties of ion-conducting channels in membranes. Elucidation of the mechanism of complement- or cell-

mediated cytotoxicity has an obvious significance for tumor immunology. Changes in membrane fluidity and cell surface mobility are considered to have important implications for cell transformation. The vesicle-lymphocyte experiments have a direct bearing upon attempts to develop drug-loaded vesicles targeted for specific tumor cells. This work supports Objectives 3 and 6 of the National Cancer Plan.

Proposed Course: The BLM will continue to be used as an assay for ionophores from natural membranes. Our next candidate for ionophoric activity is the purified IgE receptor from mast cells, since its biological activity has been associated with Ca^{2+} influx. The photobleaching technique will be applied both to surface receptors incorporated into planar bilayers and to mobility of cell surface receptors for hormones, immunoglobulins, and transplantation antigens. We also hope to measure membrane flow using this technique. The complement lysis studies will be extended to include the effect of lipid fluidity and charge. The fusion studies will be continued to resolve the specific mechanism of fusion and the nature of the saturable sites we have discovered.

Publications:

Blumenthal, R., Weinstein, J. N. and Henkart, P.: Lipid Model Membrane Studies of Immune cytotoxic Mechanisms. In Miller, M. W. and Shamoo, E. A. (Eds.): Membrane Toxicity 9th Rochester Internat. Conf. on Environmental Toxicity New York, Plenum, 1977, in press.

Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W. A.: Liposome-cell interaction: Transfer and intracellular release of a trapped fluorescent marker. Science 195: 469-492, 1977.

Rosenstreich, D. L. and Blumenthal, R.: Ionophorous activity and Murine B-lymphocyte mitogens. J. Immunol. 118: 129-136, 1977.

Blumenthal, R. and Shamoo, A. E.: Incorporation of Transport Molecules into Black Lipid Membranes. In O'Brien, R. D. (Ed.): The Receptors. A comprehensive treatise. New York, Plenum, 1977, in press.

Wolf, E. E., Schlessinger, J., Elson, E. L., Webb, W. W., Blumenthal, R. and Henkart, P.: Diffusion and Patching of macromolecules on planar lipid bilayer membrane. Biochemistry (1977) In press.

Kafka, M. S., Blumenthal, R, Walker, G. A. and Pollard, H. B. The effect of dopamine-B-hydrocylase on the electrical conductance of bimolecular lipid membranes. Membrane Biochemistry In press.

Pagano, R. and Weinstein, J. N.: Interactions between lipid vesicles and mammalian cells. Ann. Rev. Biophys. and Bioeng., 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08306-05 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Kinetic Modeling of Human Plasma Lipoprotein Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	L. Zech, M.D.	Research Associate	LTB NCI
	D. Foster, Ph.D.	Staff Fellow	LTB NCI
	C. Malmendier, M.D.	University of Brussels, Belgium	
	C. Schwartz, M.D.	Veterans Administration, Richmond, VA	
	A. Le	University of CA at San Diego	

COOPERATING UNITS (if any) Drs. H. Brewer, E. Schaefer and R. Levy, Molecular Disease Branch, NHLI, NIH; Drs. S. Grundy, Veterans Admin, San Diego, CA; Dr. W. Fisher, Univ. of Fl, Gainesville, FL; Dr. W. Hazzard, Univ. of Washington, Seattle, WA; Dr. Steinberg, UCSD, School of Medicine, San Diego, CA

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of plasma apoproteins, cholesterol and triglyceride are being constructed based on data from experiments in man. The models are used to integrate plasma lipoprotein interactions with enzymes and receptors and to provide a better understanding of plasma lipoprotein synthesis and metabolism in health and disease. The models are particularly useful for the rigorous testing of hypothesis, the design of future experiments, and in quantitating the effects of various perturbations.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of lipoproteins metabolism in man and to identify abnormalities and drug effects through the modeling of apoprotein, cholesterol, and triglyceride kinetics. Because of the need for diverse extensive data on a variety of patients, collaboration with several experimental groups is maintained.

Methods Employed: Mathematical modeling is the tool used for the integration and analysis of the data. This is performed with the help of the SAAM computer modeling program.

Major Findings: The HDL apo A model previously developed was extended to data from patients with Tangiers disease. The results of this analysis suggest that the synthesis rates for apo A proteins are near normal and degradation is enhanced (Foster). The Apo B and Apo C models previously developed have been extended to include the studies of Hazzard in Seattle and Schaefer at NHLI. Although new features are required for the model which are not yet finalized, the essential features for the previous models have been further validated. Comparison of synthesis of catabolism of VLDL between normal, obease, diabetic, and hyperlipemic groups have been made (Zech). The apo B and glycerol conversion to triglyceride models have been combined to examine a new set of data collected on both triglyceride and apo B simultaneously. This combination of models gives further support to the slow synthesis pathway for triglycerides (Le ngoz Anh).

The model for cholesterol synthesis from mevolonic acid precursors has been used to suggest new experiments separating the synthesis of cholesterol by the liver from synthesis in preferred organs (Schwartz). A new model for the delipidation of chylomicrons by bovine lipoprotein lipase was formulated. It was shown that the modulation of free fatty acids by albumin controls lypolysis of tri, di and mono glycerol steps through a common control function (Foster). Data from the study of a second patient with poly disperse LDL was analyzed and found to be consistent with our previously proposed model (with W. Fisher).

Significance to Biomedical Research and the Program of the Institute: Modeling is important to identify the abnormalities in metabolism responsible in hyperlipemics, diabetics, and other diseases, given a very complicated system with multiple interactions. It is hoped that by identifying the mechanisms responsible for abnormalities in lipid metabolism and their relations with intermediate metabolism, appropriate treatment can be more specifically geared to abnormalities. Clinically this is relavant to atherosclerosis and disorders associated with athersclorsis such as cardiovascular desease, diabetes, cachexia, and other metabolic disorders involving fat and lipids.

Publications:

Phair, D., Hall, M. III., Bilheimer, D. W., Levy, R. I., Goebel, R. H. and Berman, M.: Modeling lipoprotein metabolism in man. Summer Computer Simulation

Conference. Washington, D.C., Simulation Councils, Inc., 1976, pp 486-492.

Berman, M., Hall, M., III, Levy, R., Eisenberg, S., Bilheimer, D. W., Phair, R. D., Goebel, R. H.: Metabolism of apo-B and apo-C lipoprotein in man: Kinetic studies in normals and hyperlipoproteinemics. J. Lipid Res. 1977, In press.

Blum, C. G., Levy, R. I., Eisenberg, S., Hall, M., III, Goebel, R. H. and Berman, M.: High density lipoprotein in man. J. Clin. Invest. 1977, In press.

Schwartz, C. C., Berman, M., Vlahcevic, Z. R., Halloran, L. G., Gregory, D. H. and Swell, L.: Multicompartmental analysis of cholesterol metabolism in man. I. Characterization of the hepatic bile acid and biliary cholesterol precursor sites. J. Clin. Invest., 1977, Accepted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08312-04 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Computer Applications: Programming and Data Processing

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: F.K. Millar, Ph.D. Computer Specialist LTB NCI

COOPERATING UNITS (if any)
None

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Mathematical Biology Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop software for data processing and other computer applications in the investigation of selected biomathematical problems.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To facilitate the investigation of biomedical research problems through the application of computer programming and computer data processing.

Methods Employed: Use is made of the PDP-11/20 computer of this laboratory, the National Bureau of Standards Univac 1108 computer, and the central NIH computer facility. The projects reported may be collaborative efforts with investigators in other units, as shown below.

Major Findings:

Computer Applications:

(1) Analysis of feeding patterns in rats (Dr. S. D. Morrison, C/LPP). Data, produced as rats feed from an eatometer and collected on magnetic tape cassette, give, after appropriate corrections, direct measurement of feeding intensity which can then be used to calculate actual meal lengths and inter-meal intervals. Earlier approaches for the reduction, correction and analysis of these data by the PDP-11/20 computer are being adapted to an on-line Wang 2200 system.

(2) Processing and plotting of data from liquid spectrometers; comparison of isoaccepting species of tRNA isolated from normal and malignant cells (Dr. J. F. Mushinski, Dr. M. S. Marini, C/LCBGY).

Data collected on punched paper tape are reduced and analyzed on the PDP-11/20. Final results are plotted directly on the on-line CalComp plotter and are also stored on magnetic tape for future retrieval, e.g., for additional analysis on the NIH central computers.

(3) Further refinements have been made to the general purpose plotting package being developed for the CalComp plotter on-line to the PDP-11/20. A special purpose edit program is also under development which operates in conjunction with the plotting package. The editor provides mechanisms for building a file containing in proper sequence the necessary information to direct the production of graphs on the plotter. This should simplify the process whereby the user formulates, tests and re-designs graphs as desired. Under the edit program's default procedures, a graph of the observed and calculated data generated as output from a run of the SAAM program (Project No. ZO1 CB 08300-05 LTB) will result.

Significance to Biomedical Research and the Program of the Institute: Computer programming applications contribute to the investigation of research problems by providing (1) accurate and efficient methods for handling laborious calculations of data and visualizing results, (2) the ability to store and retrieve large amounts of data, and (3) opportunities for testing mathematical models based on experimental results.

Proposed Course: Programs will continue to be developed and computational

techniques to be applied in response to computer-related needs of the scientific staff.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08317-03 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Theory of Conformational Transitions in Polypeptides and DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Jernigan, Ph.D.	Theoretical Physical Chemist	LTB NCI
OTHER:	S. C. Szu, Ph.D.	Visiting Fellow	LTB NCI

COOPERATING UNITS (if any)
None

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0.4	0.4	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to elicit molecular details of conformational kinetics in polypeptides and DNA. An eventual aim is to understand the mechanisms and pathways of DNA double helix formation, of protein folding, and of denaturation and renaturation. A mathematical model of the rates for passing between the various configurational states was previously formulated. Molecular states are determined by specification for each residue of a helical or random coil conformation. Averages are performed over all possible conformations. Calculated results have been compared with experiments on DNA. Effects of perturbation direction and size were calculated; strong dependences were observed, mostly in agreement with corresponding experiments. The effects of including a bimolecular reaction for the formation of the first DNA helix unit were investigated; for usual conditions this effect is small.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine the molecular details of conformational kinetics in macromolecules. An eventual aim is to understand the mechanisms and pathways of protein folding and denaturation and renaturation kinetics.

Methods Employed: A mathematical model of the rate for passing between the various configurational states was previously formulated. Molecular states are determined by specification for each residue of a helical or random coil conformation. Averages are performed over all possible conformations.

Major Findings: The previous model, appropriate for polypeptides, has been extended to treat conformational relaxation in deoxyribonucleic acids. A major reason for this extension is the possibility of making direct comparisons with abundant data. Effects of perturbation direction and size were calculated; strong dependences were observed, mostly in agreement with corresponding experiments. However, disagreement was found for the rate of approach to equilibrium in denaturation. The effects of including a bimolecular reaction for the formation of the first DNA helix unit were investigated; for usual conditions this effect is small.

Significance to Biomedical Research and the Program of the Institute: A basic understanding of denaturation and renaturation of DNA and of proteins is essential for understanding cellular processes on a molecular basis. Such comprehension is necessary before detailed effects of external chemical agents can be determined.

Proposed Course: Calculations will be extended to attempt to account for occurrences of loops and misaligned pairs of strands. The effects of specific sequences of residues should also be investigated; in particular, it would be interesting to know which sequences of units would lead to either a single average relaxation time or to separate relaxation times for distinct molecular regions.

Publications:

Jernigan, R. L. and Szu, S. C.: Polypeptide Relaxation Rates with Configurationally Dependent Internal Friction. J. Poly. Sci. Symp. 54: 271-281, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08320-02 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Macromolecular Conformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Jernigan, Ph.D.	Theoretical Physical Chemist	LTB NCI
OTHER:	S. C. Szu, Ph.D.	Visiting Fellow	LTB NCI

COOPERATING UNITS (if any) T. T. Wu, M.D., Ph.D. Northwestern University
H. Bilofsky, Ph.D. Bolt, Beranek & Newman, Inc. Camb., MA
E. Kabat, Ph.D. NCI, NIH; Columbia Univ., Presbyterian Hospital N.Y.
S. Bourgeois, Ph.D. The Salk Institute for Biological Studies, CA

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1.6	1.6	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new method has been developed to calculate protein secondary conformations. Direct interatomic interactions are calculated to obtain probabilities of the various conformers. A selection process based on local equilibrium yields good predictions. Minimization of the total secondary structure energy does not yield as good results. The method suggests the relative stabilities of various structured regions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop adequate methods to determine the most significant molecular conformations. In particular, we wish to predict protein conformations from the sequences and to determine the responsible intramolecular interactions.

Methods Employed: A new method was developed to calculate relative probabilities of secondary structure regions for all positions in proteins. Sidechain atoms were taken at fixed positions, as determined by their average positions from X-ray crystal studies. Calculation of the most significant interatomic interaction energies yields probabilities of secondary structure regions. Several methods were devised to choose from this large set of possible secondary structure regions.

Major Findings: The best secondary structure predictions were not found by taking the most probable combination of structures but rather by successively choosing the most probable non-overlapping regions. With this method, the predictions compared favorably with the experimental X-ray structures. The implication is that proteins fold by selecting secondary structures in a specific sequence without large effects from tertiary interactions.

Significance to Biomedical Research and the Program of the Institute: Development of a reliable method for predicting protein conformations from their sequences would yield understanding of numerous biochemical processes.

Purposed Course: Further testing and refinement of the method are required. In particular, biochemical data on the behavior of known sequence mutants may provide meaningful tests. The same methods can in principle be applied to predict tertiary and quaternary protein structures. Elucidation of probable protein folding pathways would permit conformational identification of experimentally observed intermediate states. Physical properties of such states can be calculated in order to suggest experiments to verify such predictions.

Publications:

Wu, T. T., Szu, S. C., Jernigan, R. L., Bilofsky, H., Kabat, E.A.: Prediction of β-sheets in Immunoglobulin Chains. Comparison of Various Methods and an Expanded 20 x 20 Table for Evaluation of the Effects of Nearest Neighbors on Conformations of Middle Amino Acids in Proteins. Biopolymers. In press.

Jernigan, R. L. and Thompson, D. S.: Flexible Polymers. In O'Konski, C.T. (Ed.): Molecular Electro-Optics. Marcel Dekker, Inc., 1976, pp. 159-206.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08322-02 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Physical Chemistry of Lymphocyte Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. DeLisi, Ph.D. Theoretical Biologist (I.P.A.) LTB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mathematical models have been developed for describing the dynamical events which occur on the lymphocyte plasma membrane subsequent to interaction with antigen. The models have been simulated numerically in an attempt to understand whether these events are important in the control of the quality of antibody (i.e. affinity) produced during a response, and if so, how they exert their influence.

PHS-6040
(Rev. 10-76)

Project Descriptions:

Objectives: To develop an understanding of the relationship between the thermodynamics and kinetics of ligand cell-bound receptor interactions, and the requirements for lymphocyte activation.

Methods Employed: Mathematical Modeling

Major Findings: A wide range of response phenomona, many bearing no obvious relation to one another, can all be understood as consequence of a crosslinking requirement for B cell activation. In particular (1) small sparsely haptenated antigens such as $DNP_{10}BSA$ are expected to elicit a response that matures, and such maturation can occur even with antigenic determinant density in excess over the concentration of cellular receptors. (2) There is an optimal concentration for activation of cells with a given affinity, and therefore an optimally immunogenic dose. (3) The optimal dose is relatively insensitive to antigen valence. (4) Increasing valence increases the breadth of the affinity distribution. (5) For supra optimal doses of antigen, unresponsiveness will be preferentially induced in high affinity cells. (6) Small densely haptenated antigens (e.g. $DNP_{40}BSA$) are not expected to elicit responses that mature as quickly as those that are lightly coupled. (7) Large polymeric antigens are not expected to induce responses that mature. (8) Antigens with low determinant density may induce tolerance *in vivo* but not *in vitro*. The predictions are briefly discussed in the context of relevant experimental data.

Proposed Course: Work in the immediate future on regulatory aspects of the immune response will concentrate primarly on T independent antigens, and specifically on understanding synchronous cycles in the number of plaque forming cells with time after immunization and oscillations in affinity early in the response.

Significance to Biomedical Research and the Program of the Institute: Understanding the basis of immune recognition and control (including paralysis and unresponsiveness) would clearly be an important step in understanding conditions under which a host's immune system might be effective in eliminating or holding in check incipient tumors, and in providing a rational basis for immunotherapy. This project is a link between assay quantitation and immune surveillance models.

Publications:

DeLisi, C. and Perelson, A.: Kinetics of aggregation phenomona. I. Minimal Models for Patch Formation on Lymphocyte Membranes. J. Theor. Biol. 62: 159-210, 1976.

DeLisi, C. and Thakur, A.: Antigen Binding to Receptors on Immunocompetent Cells. II. Thermodynamic and Biological Implications of the Receptor Cross-Linking Requirement for B-Cell Activation. Cellular Immunol. 28: 416-426, 1976.

DeLisi, C.: Some Mathematical Problems in the Initiation and Regulation of the Immune Response. Math. Biosci. In press.

DeLisi, C.: Physical Chemical Aspects of Cellular Selection in an Immune Response. In Bell, G. I. (Ed.): Theoretical Immunology. New York, Marcel Dekker, 1977, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08323-02 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Quantitation of Immunochemical Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. DeLisi, Ph.D.	Theoretical Biologist (I.P.A.)	LTB NCI
Other:	A. K. Thakur, Ph.D.	Visiting Fellow	LTB NCI

COOPERATING UNITS (if any)
None

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of the hemolytic plaque assay continued, with particular emphasis on the experimental conditions which must be fulfilled for inhibition experiments to yield thermodynamic information. Work on a related problem was also initiated, viz, obtaining from equilibrium binding data, the distribution of equilibrium constants characterizing the interaction of a heterogeneous receptor population with homogeneous ligand. The problem was approached by a method of moments.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To quantitate certain immunological assays and delineate conditions under which physical chemical information can be obtained.

Methods Employed: Mathematical Modeling

Major Findings: Application of plaque inhibition theory led to the reconciliation of a wide body of apparently conflicting results on IgM affinity maturation. Specifically I found that all workers who reported absence of maturation were unknowingly using assay conditions which the theory predicts are insensitive to affinity variations, while those who reported maturation were using conditions which theory predicts are optimal or near optimal.

For the receptor heterogeneity problem, Monte Carlo simulations indicate that for a wide range of distributions, the method should yield the first two moments to within experimental error, and for distributions which do not deviate too severely from a gaussian, the method should yield the entire distribution.

Significance to Biomedical Research and the Program of the Institute: In order to develop a complete understanding of the relationship between the immune system and tumors, physical chemical aspects of their interaction must be characterized. This project is working toward that goal.

Proposed Course: The hemolytic plaque theory is currently being used, and will continue to be used in the coming year, to design experiments which will allow new information on antibody secretion rate distributions to be obtained. The work will be in collaboration with Drs. Bruce Merchant (BB,DBBP) and Gino Doria (Rome).

Publications:

Author: DeLisi, C.: Antigen-Antibody Interactions. Heidelberg, Springer-Verlag, 1976, 144pp.

DeLisi, C. and Metzger, H.: Some Physical Chemical Aspects of Receptor Ligand Interactions. Immunol. Comm. 5: 417-436, 1976.

DeLisi, C.: Hemolytic Plaque Inhibition: The Physical Chemical Limits on Its Use as an Affinity Assay. J. Immunol. 117: 2249, 1976.

DeLisi, C.: Detection and Analysis of Recognition and Selction in the Immune Response. Bull. Math. Biol. In press.

Thakur, A. and DeLisi, C.: Theory of Ligand Binding to Heterogeneous Receptor Populations Characterization of the Affinity Distribution Function. Submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08325-02 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Compartment Modeling of Complex Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. Rescigno, Ph.D. Mathematician (I.P.A.) LTB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To construct a model of a complex system involving a large number of compartments, some important parameters can be computed from the experimental data available from tracer experiments. From those parameters a model compatible with the data, but containing a minimum number of compartments is first constructed; then all possible transformations of the model that do not change the above parameters are studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To construct in the most efficient way models of complex systems with a large number of compartments.

Methods Employed: Operational calculus and theory of graphs.

Major Findings: A number of parameters, called Weight, Transfer Time, and Dispersion, can be computed in a simple way from the experimental data available from tracer experiments. Additional parameters can be obtained by computing higher moments of the transfer function. From these parameters the connectivity of the system and its transfer rates can be derived.

Significance to Biomedical Research and the Program of the Institute: Modeling of complex systems is essential to the understanding of normal and pathological states. This project closely fits Objective 4, Approach 4 of the National Cancer Plan.

Proposed Course: The problem to be solved now is to estimate the error in the determination of the moments of the transfer function, due to the fact that the tracer is measured only for a finite interval of time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZOL CB 08326-02 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Dynamical System Theory in Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. Rescigno, Ph.D. Mathematician (I.P.A.) LTB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The non-linear differential equations describing the behavior of a population of molecules, or of cells, or of individuals, are studied in the context of systems theory. Necessary and sufficient conditions for the existence of enclosures are sought, and their biological importance investigated.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To study the general properties of the non-linear differential equations describing the behavior of a population of molecules, or of cells, or of individuals, subject to different kinds of interaction.

Methods Employed: The primary mathematical tool used in dynamical systems theory is the theory of autonomous non-linear differential equations.

Major Findings: The major results are the necessary and sufficient conditions for the existence of enclosures and for the presence of oscillations with some kinds of interactions.

Publications:

Rescigno, A.: The Struggle for Life - IV. Two Predators Sharing a Prey. Bull. Math. Biol. 39: 179-185, 1977.

Rescigno, A.: The Struggle for Life - V. One Species Living in a Limited Environment. Bull. Math. Biol. 39: 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08327-02 LTB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical Methods for Analysis of Large Scale Metabolic Networks.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.J.M. Park Expert LTB NCI

COOPERATING UNITS (if any)

None

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NCI, NIH, Bethesda, Maryland 20014

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01	01	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project concerns methods for mathematical analysis of metabolic networks based upon stoichometric techniques and hierarchical structure. Efficient computational methods for solving equilibrium subnetworks, some steady state subnetworks, partial inverse equilibrium and the initial mix (or assay) problem have been developed.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop mathematical methods which will aid in the analysis, simulation and conceptual understanding of large scale metabolic systems. The development of metabolic network theory.

Major Findings: Many mathematical methods have been developed which aid in the analysis of metabolic and chemical systems. The methods offer computational efficiencies and also aid in the conceptual understanding of metabolic systems. A computer program, MACE, was developed for the solution of general mass action chemical equilibrium problems of any size. It uses an easy chemists's style input.

Significance to Biomedical Research and the Program of the Institute: The methods developed have an across-the-board application to all problems which depend upon facility in the mathematical analysis of biochemical systems.

Publications:

Park, D. J. M.: Numerical Methods for Solving the Chemical Mass Action equilibrium Problem. J. Chem. Phys. 65: 3085-3091, 1976.

Park, D. J. M.: Simple Methods for Calculating Reaction Equilibria in Single Reaction or Single Moiety Chemical Networks. Computer Programs in Biomedicine. 6: 263-268, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08329-01 LTB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical Modeling of Renal Medullary Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. M. Foster Sr. Staff Fellow LTB NCI

COOPERATING UNITS (if any)

J. A. Jacquez, Dept. of Physiol., University of Michigan

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NCI, NIH, Bethesda, Maryland 20014

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0.2	0.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A model of the countercurrent concentrating mechanism in the renal medulla previously developed has been used to study the medullary function of the rabbit and rat.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: To incorporate into the model all of the available parameters descriptive of salt and urea transport in the renal medulla and, using these, to test the model performance against the various hypothesis as to the details of the concentrating process in the rabbit and rat.

Major Findings: The model for the rabbit was consistent with the experimentally determined values for salt and water movement, but was not consistent with the data on urea. This suggests that there is a possibility of some form of mediated transport for urea in the inner medulla. The model for the rat was not consistent with the experimentally determined values suggesting that the parameter values which were estimated from micropuncture studies are in error.

Proposed Course: To refine the model to include individual ion transport for both sodium and chloride, to add the provision of a urea pump in the collecting duct.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08330-01 LTB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mathematical Models of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. DeLisi, Ph.D. Theoretical Biologist (I.P.A.) LTB NCI

COOPERATING UNITS (if any)

L. Liotta, M.D., Ph.D. LP, NCI, NIH

LAB/BRANCH

Laboratory of Theoretical Biology

SECTION

Mathematical Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work in this area attempts to formulate a broad conceptual framework within which one can incorporate as much of the phenomonology of carcinogenesis as possible, and to develop mathematical models for specific aspects of carcinogenesis within this framework, that lead to testable predictions or suggestions for original experiments. Efforts are currently directed toward both fundamental and applied theory. The former category presently includes models of the host defense interacting with the primary tumor, and models of metastasis formation. In the latter category a specific model for breast cancer epidemiology has been proposed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop mathematical models which describe and lead to a fuller understanding of factors influencing the age incidence of female breast cancer, the dynamics of metastasis formation and aspects of the interaction of the immune system with tumor associated antigens.

Methods Employed: Mathematical Modeling.

Major Finding: The breast cancer model fits a wide range of epidemiological data and accounts at once for the several age incidence patterns observed world wide; viz, a continuous increase in incidence with age; an increase with a single interruption at menopause and an increase which continues until the menopause and then declines. One clear prediction of the theory is that in Japan the effective at risk population is only about two percent of the total population, whereas in Western countries the figure is about an order of magnitude larger. Carefully planned genetic studies will be required to test this. The model is also able to simulate the effect of various risk factors on incidence and here too, leads to new predictions.

The work on metastasis has been part of a collaborative effort with Dr. Lance Liotta, Lab. of Pathology, DCBD. We have shown that all available experiments which report the dynamics of artificial metastases, with and without treatment of the host, can be fitted by a two compartment model, the first compartment representing cells which have lodged in the vessels of the target organ, and the second, cells which have penetrated the tissue.

The model of tumor cell - host defense interaction indicates that even with a simple two variable non linear system, much of the apparent irregularities in the literature may be simulated. It may thus be crucial to consider biological variability within the context of the dynamical structure of the system, in trying to understand the host response to tumor associated antigen.

Proposed Course: The work on tumor-host interactions will be terminated because of lack of personel support. The work on metastasis will be extended to take account of their size distribution, and perhaps distance from the primary tumor, and will be integrated with the breast cancer model.

Significance to Biomedical Research and the Program of the Institute: Understanding the factors which influence the growth and spread of malignancies is a central problem in cancer research and the goal of our effort is to develop such an understanding by using mathematical modeling to analyze experimental data.

Publications:

DeLisi, C. and Rescigno, A.: Immune Surveillance and Neoplasia. I. A Minimal Math Model. Bull. Math. Biol. 39: 201-211, 1977.

Rescigno, A. and DeLisi, C.: Immune Surveillance and Neoplasia. II. Bull. Math. Biol. In press.

Liotta, L. A., DeLisi, C., Saidel, G. and Kleinerman, J.: Micrometastasis Formation. A Probabalistic Model. Cancer Letters. In press.

Liotta, L. A., Saidel, G., Kleinerman, J., DeLisi, C.: Micrometastasis Therapy Theoretical Concepts. In Day, S. B. (Ed.): Proc. Cancer Metastasis Workshop. New York, Raven Press, 1977, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08331-01 LTB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Glucose-Insulin Kinetics Modeling of Carbohydrate Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Berman, Ph.D.	Acting Chief	LTB NCI
OTHERS:	E. A. McGuire, Ph.D.	Staff Fellow	LTB NCI
	D. Foster, Ph.D.	Staff Fellow	LTB NCI

COOPERATING UNITS (if any)
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Dr. J. Roth, Diabetes Branch, NIAMDD, NIH, Bethesda
Dr. G. Hetenyi, Jr., Dept. of Physiology, Univ. of Ottawa

LAB/BRANCH
Laboratory of Theoretical Biology

SECTION
Mathematical Biology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic models of the glucose regulatory systems are being developed for man and other animals in collaboration with several experimental groups. These include models of the distribution, metabolism, and receptor binding of insulin using data both radioiodinated and native insulin models of the production and utilization of glucose as elucidated by tracer studies with labeled glucose alanine and lactate, and models of the role of insulin in the control of glucose utilization and production. The models are being used to gain insight into the metabolic changes which take place in various conditions of altered glucose homeostasis such as obesity and diabetes.

Kinetic models of the glucose regulatory system, including the insulin subsystem, are being developed for man and other animals from both collaborative and literature data.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop a qualitative and quantitative understanding of glucose homeostasis including the role which various hormones insulin plays in the production and utilization of glucose and the role which glucose plays in the secretion of insulin in normal and perturbed metabolic states.

Major Findings: The insulin model for young normal males has been applied to males covering a wide range of age and obesity as well as to those with maturity onset diabetes. Findings include a decrease in the tissue to plasma insulin mass ratio with obesity and diabetes but an increase with age. These changes most probably are due to similar changes in binding of insulin to its receptors. The magnitude of the changes in the insulin subsystem is not sufficient to account for the observed glucose intolerance seen in these three states, however, the changes seen with age are in opposite direction from those seen with maturity onset diabetes suggests that diabetes is an entity distinct from the normal aging process.

A more detailed understanding of the insulin subsystem also is being obtained from the modeling of radioiodinated tracer data on different species of insulin which have either low or high affinity for the insulin receptor in rabbits. Tentative findings include a 3 compartment model to fit the low affinity (presumably non-receptor binding) insulin with the requirement for 2 additional compartments to fit the high affinity (presumably receptor bound) insulin. This suggests 2 states for bound insulin (one more tightly bound than the other) and suggests a method for the measurement of insulin bound to receptors in vivo.

The model developed earlier by Hall et al for the flow of alanine carbon atoms into glucose in postabsorptive dogs is being extended to include new data measuring directly the flow of alanine carbon into lactate and the flow of glucose carbon back into alanine and lactate by use of ^{14}C and ^{3}H labeled tracer (Foster). The studies have been performed in pancreatectomized adult dogs as well as in normals. This extended model will add more detail to our understanding of the production and utilization of glucose in the postabsorptive state in normal and diabetic-like dogs.

The model developed to account for the differences in glucose level between simultaneously obtained arterial and venous blood samples will permit the analysis of a large body of data on glucose control of insulin secretion.

Significance to Biomedical Research and the Program of the Institute: Carefully tested models provide predictions as to how a complicated regulatory system will respond to a given perturbation. The clinically or experimentally observed responses seen in various altered metabolic states can thus be analzed using such models to gain insight into the basic perturbations which have accurred in the states. Obesity, diabetes, aging and the cachexia of advanced cancer are examples of metabolic conditions in which glucose homeostasis is perturbed and which can thus be better understood by use of these models.

Proposed Course: 1) To study the regulatory behavior of the glucose-insulin system in an attempt to identify the primary perturbations in the obese, diabetic and aged subjects for which data are available. 2) To extend the insulin model to account for differences between studies using radioiodinated and native insulin, and to include new information on the binding and degradation of insulin. 3) To study the biological effects of insulin action.

Publications:

Hall, Susan, E. H., Goebel, R., Barnes, I., Hetenyi, G., Jr. and Berman, M.: The Turnover and Conversion to Glucose of Alanine in New-born and Grown Dogs. Fed. Proc. 36: 239-244, 1977.

McGuire, E. A. H., Helderman, J. H., Tobin, J. D., Andres, R. and Berman, M.: Effects of Arterial Versus Venous Sampling on Analysis of Glucose Kinetics in Man. J. Appl. Physiol. 41: 565-573, 1976.

SUMMARY REPORT

IMMUNOLOGY BRANCH

Fiscal Year 1977

The Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis given to studies of regulation and control of immune responses, structure and function of cell surfaces, transplantation biology and tumor immunology. In addition, clinical studies of relevance to tumor immunology are performed.

A. REGULATION AND CONTROL OF IMMUNE RESPONSES

It has been known for some years that specific antibody synthesis is controlled by genes located in or near to the major histocompatibility complex (MHC). The mechanism of this genetic control has not yet been precisely defined, at least in part because there have not been in vitro systems suitable for appropriate detailed analysis. An in vitro system for generating both primary and secondary antibody responses to soluble antigens has therefore been developed. Results obtained with this system indicate that there exists genetic control of IgM as well as IgG responses and that the macrophage may be the cell that actually confers genetic regulation (CB-05064).

Genetic control is also being studied in vivo, utilizing the complex antigen staphylococcal nuclease. Previous studies had shown that antibody responses to this antigen are under control of H-2-linked immune response genes. Antibodies against idiotypic determinants on the antinuclease antibodies have been produced in rats. These reagents have been used to screen a variety of strains for the presence of idiotype. These studies led to the conclusion that idiotype expression is linked to the heavy chain allotype marker, rather than to the immune response gene loci (CB-05036).

Viruses and some chemicals can modify the surface of cells to create new antigens that depend both on the modifying agent and on the histocompatibility antigens of the cell surface being modified. TNP-modified spleen cells induced cytotoxic effector cells when used to stimulate syngeneic lymphocytes in vitro. The effector cells only lysed TNP-modified target cells if the target cells shared appropriate H-2 antigens with both the stimulating and effector cells. This immune response itself is under the genetic control of genes that are H-2-linked (CB-05038). Similar findings have been made with human lymphocytes (CB-05067). The fine specificity of the cell-mediated immune response in mice has been studied with a series of related nitrophenyl compounds (CB-05045). Antibody production to TNP-modified syngeneic lymphocytes was also studied in a number of mouse strains. Differences were found between the requirements for antibody production and for generation of cytotoxic effector cells, leading to the suggestion that antibodies and T cell receptors recognize different antigenic specificities (CB-05066).

Functional and serologic characterizations of cells important to the immune response have continued. Studies of the distribution of Ia antigens on various cell populations have shown that spleen macrophages express more I-region antigens than do peritoneal macrophages (CB-05065). Peritoneal adherent cells, usually thought to be mostly macrophages, were shown to contain a small subpopulation of adherent B cells with potent anti-tumor activity (CB-05054). Antisera directed against I-region determinants abrogated T cell proliferation to Con A but not to PHA (CB-05069). The cells responsible for hemopoietic graft rejection in vivo, F_1 anti-parent cytotoxicity in vitro and "natural" killing have been compared and shown to differ in some characteristics (CB-05049).

Adherent accessory cells (macrophages) are required for the generation of cell-mediated cytotoxicity against allo-antigens in vitro. It has been shown that certain tumor cells, even ones not resembling macrophages, can serve as accessory cells (CB-05070).

Further attempts to identify and characterize antigen-specific T cell factors have been deferred to permit the development of a reliable in vitro assay system. Such a system has now been developed (CB-05064) and work on T cell factors will be resumed.

B. STRUCTURE AND FUNCTION OF CELL SURFACES

Many subpopulations of murine and human lymphocytes contain Fc receptors. Human B cells were shown to differ from human UL-cells in that the Fc receptors of UL-cells had much higher avidity for Ig complexes (CB-05034).

In addition, it has been shown that Fc receptors of B and T cells, but not UL-cells or macrophages, are associated with Ia antigens. A subpopulation of UL-cells, responsible for antibody dependent cell-mediated cytotoxicity, were shown not to express Ia antigens. Fc receptors and Ia antigens are separate molecules on B cells and the apparent association appears to be ligand induced (CB-05035). Continued attempts have been made to isolate Fc receptors (CB-05048). Analysis of binding affinity to the Fc receptor led to the conclusion that in vivo, only large antigen-antibody complexes should be bound (CB-05050).

Surfaces coated with anti-immunoglobulin have been used to separate lymphocytes with high concentrations of surface Ig (B cells) from other populations. Analyses of these populations indicated that human B cells do not have avidly binding Fc receptors (CB-05061).

The interaction of mouse spleen cells with surface-immobilized antigen-antibody complexes caused an elevation of intracellular cyclic-AMP (CB-05060). Other mitogens did not cause changes in cyclic-AMP and none of these manipulations caused changes in the level of cyclic-GMP.

Studies of the mechanisms whereby lymphocytes damage target cell membranes are in progress. Attempts are being made to measure intracellular electrical events during cell killing and to use liposomes as model systems (CB-05018).

Amphipathic macromolecules have been synthesized and used as models for membrane proteins. Lateral diffusion rates for such molecules have been determined and the conditions required for patching and capping ascertained (CB-05068).

C. TRANSPLANTATION

Analyses of antigens important for transplantation are being carried out in mice (CB-05021, CB-05056), swine (CB-05023), humans (CB-05006, CB-05017) and monkeys (CB-05011). Miniature pigs have been bred for homozygosity at the MHC and organ transplantation studies have been initiated. Purification and characterization of the MHC antigens are in progress. Anti-idiotype antibodies prepared against anti-histocompatibility antibodies are being investigated to test possible effects on graft rejection. The biologic significance of human histocompatibility antigens is being studied in terms of correlations between HLA haptotype and disease resistance or

D. TUMOR IMMUNOLOGY

The nature of immune responses to murine and human tumors continue to be analyzed (CB-05051, CB-05003). Analyses of AKR leukemia variants has provided additional information about murine lymphoid cell surfaces (CB-05043, CB-05053).

A clinical trial of the use of BCG or BCG plus immunization with allogeneic, cultured melanoma cells has been continued (CB-05033, CB-05016). More than 100 patients have been entered but the follow up is too short to permit a meaningful an evaluation.

E. MACROMOLECULAR BIOLOGY SECTION (OFFICE OF THE ASSOCIATE DIRECTOR FOR IMMUNOLOGY)

This Section carries out a program exploring the nature of transformed cells. SV-40-induced transformants have been studied to determine surface antigenic changes (CB-05544, CB-05526). Additional studies utilize the approach of labeling and modifying cell membrane glycoproteins with fluorescent dyes to ascertain changes that may correlate with transformation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05003-12 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

In Vitro Tests of Immunity in Patients with Melanoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
OTHER:	C. I. Civin	Clinical Associate	I NCI
	N. A. Dorfman	Visiting Fellow	I NCI
	R. I. Fisher	Senior Investigator	M NCI
	J. F. Jones	IPA Investigator	I NCI
	D. M. Segal	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Tumor immunogenicity has been increased in a mouse model system by chemical modification of the tumor cells.

2) Using Peripheral blood lymphocytes as responder cells, freshly explanted human ovarian cell carcinoma cells function well as both an alloimmunogen and target cells in cell-mediated cytotoxicity assays.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity in both humans and mice. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation, and expression of cytotoxic cellular immune responses; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes. Immediate objectives of the past year have been to concentrate on goals #2 and #3.

Methods Employed: Cell-mediated cytotoxicity is measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Immune cells are collected from spleen, marrow, lymph node, thymus and peripheral blood of sensitized hosts. Several techniques have been used for fractionating cells. Peripheral blood cells (30-40 mls whole blood) and spleen cells (mouse) have been fractionated with Ficol-hypaque gradient centrifugation and on nylon columns; effluent cells from the nylon columns are highly enriched for T lymphocytes. In addition, immune cells have been produced by in vitro sensitization of spleen and peripheral blood cells using previously established Mishell and Dutton tissue culture techniques. Target cells are provided by freshly explanted cells, tissue culture lines, and established tumor passed in vivo or in vitro; most target cells function well if previously frozen in liquid nitrogen. Target cells have also been chemically modified in order to change the immunogenicity of cell surface antigens.

Major Findings: 1) A methylcholanthrene-induced mouse tumor cell line which is not immunogenic in vitro has been treated in vitro with tetranitromethane. Normal spleen cells sensitized in vitro with this modified syngeneic tumor can generate T cells cytotoxic for the unmodified tumor cells. Cytotoxic cells crossreact with some unrelated tumors not with others; specificity of the cytotoxic reactions is as yet unknown. Chemical modification of the tumor itself is required for changes in immunogenicity, and these changes cannot be accounted for by chemically induced "helper" determinants on tumor cells. Tetranitromethane appears more effective than modifications previously used to increase tumor immunogenicity (e.g. sulfanilic acid).

2) In collaboration with Dr. R. Fisher (Medicine Branch, NCI) promising procedures for rendering mouse tumor cells immunogenic are being adapted to human tumor cells. Ascites fluid from patients with ovarian cell carcinoma or other types of tumor is normally withdrawn as part of the clinical management. Tumor cells from aliquots of ascites fluid function well in cytotoxicity assays and are immunogenic for normal allogeneic peripheral blood lymphocytes. Although peripheral blood cells from two of four ovarian cell carcinoma patients have responded to xenoantigens, cytotoxic cell responses to autologous tumor cells have not yet been induced.

Significance to Biomedical Research and the Program of the Institute: Chemical modification of tumor cells is a promising step towards stimulating cell mediated immunity against syngeneic and autologous tumors both in animal model systems and in humans. Combining this procedure with nylon-column fractionation of responding cells in order to remove suppressor cells appears to be our optimal approach toward generating anti-tumor immunity in vitro, which will be useful not only for understanding the nature of cellular immunity to tumor cells but also for passive immunotherapy.

Proposed Course of Project: A three-pronged effort will be carried out for modifying tumor immunogenicity during the next year: 1) Primary chemically induced tumors and early passages of tumor cell lines will be tested for expression of xenotropic and ecotropic murine leukemia virus antigens in order to determine whether early passages but not primary tumors bear such antigens. 2) Primary tumors and those early passaged lines which appear not to have acquired surface antigens will be chemically modified in an attempt to increase their immunogenicity. 3) The most promising procedures for increasing immunogenicity of murine tumors will be applied to human ovarian cell carcinoma tumors which will then be used to sensitize in vitro nylon column fractionated peripheral blood lymphocytes from autologous tumor bearing donors.

In addition we will continue our study of human cells which mediate antibody dependent cytotoxicity.

Publications

Eggers, A. A.: Tumor specific immunogenicity induced by chemical modification. J. Natl. Cancer Inst. 56: 869-870, 1976.

Handwerger, B. S., and Koren, H. S.: The nature of the effector cell in antibody-dependent cell-mediated cytolysis: The cytotoxic activity of murine tumor cells and peritoneal macrophages. Clin. Immunol. Immunopathol. 5: 272-281, 1976.

Eggers, A. A.: Immune response to an allogeneic progressively growing solid tumor. Cancer Res. 36: 2085-2089, 1976.

Yust, I., Smith, R., Wunderlich, J., and Mann, D.: Temporary inhibition of antibody-dependent, cell-mediated cytotoxicity by pretreatment of human attacking cells with ammonium chloride. J. Immunol. 116: 1170-1172, 1976.

Steinmuller, D., and Wunderlich, J.: The use of freshly explanted mouse epidermal cells for the in vitro induction and detection of cell mediated cytotoxicity. Cell. Immunol. 24: 146-163, 1976.

Steinmuller, D., Rodman, S. A., and Wunderlich, J.: Epidermal cells as targets and stimulators of allograft immunity in vitro. Transplant. Proc. 9: 1183-1185, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05006-12 I

PERIOD COVERED

July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Lymphocyte Cell Surface Antigens, Normal and Neoplastic

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Mann	Senior Investigator	I NCI
OTHER:	P. J. Romano	Guest Worker	I NCI
	B. Knudsen	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biologic and chemical characteristics of lymphoid and cell surface antigens are under investigation. A method for rapidly isolating the B lymphocyte subpopulation of human peripheral blood lymphocytes has been developed allowing genetic characterization of B lymphocyte antigens. Genes controlling these antigens are linked to genes controlling the HLA antigens. The B cell antigens have been found on Ig-bearing cells and are not demonstrable by cytotoxicity tests on T cells or the Fc positive K cell population. B-cell antigens isolated by immunoprecipitation techniques have been found to have two molecular components having a molecular weight of 29,000 and 34,000 daltons. Analysis of B-cell antigen frequencies in several disease conditions have been performed. A B-cell antigen identified and specific for the diseases gluten sensitive enteropathy and dermatitis herpetiformus has been found to be under the control of a gene or genes not linked to the HLA complex and appears to be a receptor for gluten, the disease causing agent. Forty N-terminal amino acids of the HLA antigens of the A locus and the B locus demonstrate near complete homology. Amino acid sequence analysis of the B cell antigens demonstrate distinct differences from the HLA antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of these studies are as follows: a) to isolate and purify human cell surface antigens for molecular and biochemical characterization; b) to develop serologic reagents to detect B cell antigens; c) to relate the presence of cell surface antigens to disease states; d) to assess the biologic function of different cell surface structures in relationship to the immune function of the lymphocytes.

Methods Employed: ^{51}Chromium release and dye exclusion cytotoxicity tests are performed for the evaluation of the presence of cell surface antigens both on normal lymphocytes, subpopulations of lymphocytes and neoplastic lymphocytes. Cells are obtained from normal donors, donors with various disease conditions, and long term human lymphoid tissue culture cell lines. A unique method for the isolation of the B-lymphocytes was developed based on the presence of surface immunoglobulin on these cells. Antiserum to the Fab component of human IgG was raised in goats. Specific antibody was purified using immunoabsorbent columns. The purified antibody is allowed to incubate on the surface of tissue culture flasks. The flasks are washed and human peripheral blood lymphocytes isolated by Ficoll-hypaque sedimentation and depleted of macrophages with carbonyl iron are layered onto the anti-immunoglobulin bearing surface. After a suitable period of incubation the non-adherent cells are removed (T and K cells) and the adherent cells or B lymphocytes are removed by competitive inhibition using normal human serum. Both the T and K cell population and the B cell population are then tested for cell surface antigens in microcytotoxicity studies. Serologic reagents used to detect these antigens have been obtained from multiparas or are heterologous sera raised by purposeful immunization.

This technique of separation of cells is also being employed to investigate the function of the different subpopulations of human peripheral blood lymphocytes in in vitro test systems. Subpopulations of cells are isolated by the method described above as well as the separation of the K cells or Fc positive cells on Falcon plastic flask coated with antigen antibody complexes. This latter technique allows the removal of the Fc positive cells. Thus 3 cell populations, the T cells, the Fc positive K cells and the B cells are obtained for in vitro studies. The various subpopulations are used in assays to differentiate the functional properties of the subpopulations of cells in the stimulation of allogeneic cells in mixed lymphocyte reaction (MLC), and as stimulators or responder cells in allogeneic mixed lymphocyte reactions (CML). In both the MLC and the CML studies the subpopulation is cultured together with either a whole population of lymphocytes from an allogeneic donor or the various subpopulations. The stimulating cells are treated with mitomycin to inhibit DNA synthesis. After a suitable incubation period the cultured cells are pulsed with tritiated thymidine and the assessment of tritiated thymidine incorporation is then determined. In similar reaction mixtures, the effects of various subpopulations on the generation of cytotoxic lymphocytes is being assessed. In these studies the cultured cells are mixed with appropriate ^{51}chromium labelled target cells and after a four hour incubation assessment of release of chromium provides information as to the generation of cytotoxicity in the responder population.

Studies of the molecular nature of the B lymphocyte antigens were performed using chronic lymphocytic leukemia cells possessing B lymphocyte surface antigens. Cells were grown in the presence of tritiated leucine for 48 hrs. Surface membrane proteins were solubilized with the non-ionic detergent NP40. The antigens were isolated on lectin columns and eluted with alpha-methylmannose. The isolated tritiated labelled antigens were incubated with alloantisera reacting with the particular cell from which the antigen was obtained or control sera. The Cowan strain Staph. aureus organism was used to isolate the antigen-antibody complex which was subsequently dissociated with SDS and electrophoresed on polyacrylamide gels containing SDS and a reducing agent mercaptoethanol. After a suitable period of electrophoresis the gels were sliced and the gel slices counted in a liquid scintillation counter. The migration of the labelled proteins was used to assess the molecular nature of the B cell antigens.

Large quantities of lymphoid cells needed to pursue the chemical and structural characterization of the HLA antigens was provided by NCI contract NO1-CB-12078. HLA antigens are solubilized with nonionic detergents or papain and the isolated antigens purified on immunoabsorbent columns, sizing and ion exchange chromatography. The isolated HLA alloantigen is assessed for activity in assays using antisera recognizing a particular specificity. The inhibition of cytotoxicity using the solubilized material provides the means for the assessment of the presence of the antigen. The antigens determined by the A and B locus are separated by DEAE chromotography. Amino acid sequencing is performed on the Beckman 890B automatic sequencer. Carbohydrate analysis is performed using gas chromatography. The above studies were performed in collaboration with Dr. Jack Strominger, Harvard Biological Laboratories, Cambridge, Mass.

Major Findings: Sixty antisera identifying the specific B cell antigens have been procured and are available for typing for B cell antigens. These sera are specific for B cell antigens, the majority of these sera have been absorbed with pooled platelets to remove any or all associated HLA reactivity. These sera have been used to study the relationship of the genes controlling the B cell antigens to the genes controlling the HLA-A and HLA-B antigens. Three family studies have been performed where one individual in the family demonstrated a cross-over between the HLA-A and HLA-B loci. These studies suggest that genes controlling expession of some B cell antigens follow the A locus while the expession of other B cell antigens follow the HLA-B determinant.

These antisera have been used to assess the presence of B cell antigens on cells frozen and thawed and compared with freshly obtained peripheral blood lymphocytes. The results indicate that B lymphocytes can be isolated from whole peripheral blood lymphocyte populations maintained in a frozen state and thawed at the time of use. Comparable results were obtained from frozen and fresh cells demonstrating that frozen cells can be used for B lymphocyte antigen determination. /

Studies have continued in assessing of the reactivity of B cell antisera in patients with several disease conditions. Studies have been performed in patients with gluten sensitive enteropathy and dermatitis herpetaformis. B lymphocyte antigens specific for the above diseases have been identified. In screening a large panel of normal individuals this antigen appears in approximately 3% of the normal population. Family studies wherein at least one individual in a sibship or parent had gluten sensitive enteropathy has clearly demonstrated that the gene controlling the expression of this antigen is not linked to the major histocompatibility complex and indeed is probably on another chromosome. These family studies also strongly suggest that homozygosity for the gene controlling the expression of this antigen is necessary for the expression of the disease. Additional studies have been performed to attempt to inhibit the reaction of these antisera against B lymphocyte antigens with the wheat germ protein, gluten, the causative agent for this particular disease. The results thus far suggest that the B lymphocyte antigen detected by the above antisera is reacting with a receptor for the wheat germ protein.

Studies have been performed in 17 patients with Sjogren's syndrone. Four B cell antigens appear to be more frequent in this disease condition in comparison to a population of normal individuals comprising tests on approximately 100 individuals. Studies have been performed in patients with diabetes mellitis. Several B cell antigens also appear to be more frequent in this particular disease condition in insulin dependent diabetics as compared to the normal population.

The presence of B cell antigens was assessed on the peripheral blood cells from patients with acute lymphocytic leukemia, acute mylocytic leukemia and erythro-leukemia. It was found that the blast cells (or malignant cells) from these patients possessed a variety of B cell antigens as demonstrated by their reactivity with the cytotoxic antisera detecting normal peripheral blood B cell antigens. The most extensive study was performed in patients with acute lymphocytic leukemia. In these studies the presence of B cell antigens was compared on three categories of acute lymphocytic leukemia (ALL), the so-called T cell leukemias and two categories of so-called null cell leukemias. This latter category can be divided into cells that stimulate allogeneic cells in mixed lymphocyte culture and those not demonstrating mixed lymphocyte stimulation. A variety of B lymphocyte antigens were demonstrated on all ALL cells. There was no particular pattern of predominance of reactivity of one antiserum or another in these studies. The significance of these results relates to two questions. One is the fact that B lymphocyte antigens are expressed on ALL T cells indicating that the means for expression of these antigens may be found in all stem cells of the lymphoid series. The other important implication of this investigation is that B cell antigens were present in cells which did not stimulate allogeneic cells in mixed lymphocyte culture. A number of reports have suggested that the HLA-D locus is responsible for mixed lymphocyte reactions, and, in addition that this particular locus is responsible for the expression of B lymphocyte antigens. This study using the lymphocytic leukemia cells wherein it was observed that cells possessing the B lymphocyte antigens did not stimulate mixed

lymphocyte culture indicates that these antigens are not solely responsible for that particular in vitro biologic phenomena.

The B lymphocyte alloantisera have also been used in precipitation studies to determine the molecular nature of the cell membrane component bearing the B lymphocyte antigen determinants. These studies performed with chronic lymphocytic leukemia cells demonstrated that the alloantisera precipitate a component or components the size of 34,000 and 29,000 daltons. These studies demonstrate the homology of the Ia antigens in mice and the B lymphocyte antigens in man. Thus we have demonstrated that the B lymphocyte antigens are controlled by genes linked to the major histocompatibility antigens, that these antigens are found primarily on B lymphocytes. The molecular component bearing the B lymphocyte antigens is similar in size to the murine Ia antigens.

Studies were also performed to determine the specific subpopulations of cells with which these sera were reacting in cytotoxicity tests. Studies have demonstrated that the B cell antigens are most easily detectable on the Ig bearing B lymphocytes and do not appear to be present at least in detectable quantities (by complement dependent cytotoxicity reactions) on the T cells or the Fc bearing K or null cells. Studies have been performed to determine the in vitro biologic reactivity of the three subpopulations of cells mentioned above. In these studies, lymphoid tissue culture cell lines were used as stimulating cells. These cell lines used were in pairs being derived from the same individual, one particular cell line having T cell characteristics the other cell line having B cell characteristics. It was demonstrated that both B and T lymphoid tissue culture cell lines can stimulate in mixed lymphocyte culture with allogeneic cells, however, only the B lymphocytes generate cytotoxic reactivity at the allogeneic cells.

Milligram quantities of the HLA-A gene product and the HLA-B gene product have been isolated from lymphoid tissue culture cell lines. These studies are performed in collaboration with Dr. Jack Strominger of the Harvard Biologic Laboratories in Cambridge, Massachusetts. Amino acid sequence studies of the products of these two genes have thus far demonstrated complete homology with approximately one third of the molecule sequence. Partial sequence studies are now performed on the HLA antigens on four different HLA antigen products of these two gene loci. In addition, the B lymphocyte antigen sequence studies have been initiated using B lymphocyte antigens from two different sources which represent different B lymphocyte antigens. These studies have demonstrated a difference in the amino acid sequence of the 34,000 molecular weight component compared to the 29,000 molecular weight component obtained from the same individual. However, in comparing the antigens derived from two different sources, there appears to be homology in the amino acid sequence of the 34,000 molecular weight component comparing two individuals and also homology in the amino acid sequence of the 29,000 molecular weight component from two different individuals.

Significance to Biomedical Research and the Program of the Institute: The human B lymphocyte antigens appear to be analogous to the B cell alloantigenic system in mice. Murine B lymphocyte alloantigens appear in some way to

be associated with the ability of the animal to respond to particular antigen. The identification of B cell antigens in man allows similar investigation in the assessment of this immunologic phenomena. A variety of disease states are associated with an increased frequency of certain HLA antigens. This association has not been complete in that not all individuals having a particular HLA antigen have a disease with which it is associated and conversely not all individuals with a disease have a particular HLA alloantigen. This suggests the possibility that another marker, which may be associated with an immunologic response is involved with the pathogenesis of the disease. B cell alloantigen typing provides the opportunity for the assessment of such a marker. The rather extensive studies in gluten sensitive enteropathy and dermatitus herpetaformis provide a prototype for the investigation of disease states associated with the expression of the B lymphocyte antigens. The cell membrane structures of human lymphocytes may serve as receptors for the initiation of the immune response or participate in the efferent limb of immune reactivity. Identification of these receptors as well as determination of biologic activity are important fundamental biologic questions.

Structural analysis of the HLA alloantigens approaches several biologic questions. The protein structure of the cell membrane components of lymphocytes is unknown. The HLA antigenic markers provides a resource for the isolation and purification of specific cell membrane structures. The structural analysis of cell membrane components will provide information as to the nature of differences in membranes that determine their individual antigenicity. Cell membrane structural changes as well as antigenic changes are thought to occur in neoplasia. Therefore structural studies on normally determined genetic products provide a prototype for studies of antigenic changes that occur in neoplastic transformation.

Proposed Course of Project: Studies will be continued in the determination of B lymphocyte alloantigens. These studies are directed at the mapping of the genes controlling the expression of these antigens. A continuing effort will be made to procure more antisera to be used in these studies. The assessment of the presence of B cell antigens on the B lymphocytes from a patients with a number of disease states is underway and will be continued throughout this next year.

Studies will be continued in an attempt to demonstrate some immunologic or biologic function of the B cell antigens. The in vitro biologic function of subpopulation of normal human peripheral blood lymphocytes will be studied. Amino acid sequence studies will be continued on the isolated purified HLA-A and HLA-B products. In addition, structural analysis will be continued on the B lymphocyte specific antigens. Attempts will be made to correlate molecular structure with the property of antigenicity.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05011-09 I

PERIOD COVERED

July, 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Rhesus Monkey Histocompatibility Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. R. Neefe, Jr. Investigator I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rhesus monkey alloantisera raised by planned immunization were evaluated for lymphocytotoxic antibodies by (1) titration against peripheral blood lymphocytes of selected monkeys; (2) typing of random unrelated monkeys and of monkey families by standard microcytotoxicity methods; (3) absorption analysis. Such methods have resulted during the past year in an increase of RhLA serologically defined antigens which we recognize from 18 to 21 including 13 in the A series and 8 in the B series.

Two monkeys have been identified which behave as LD typing cells or homozygotes at the major locus determining MLC stimulation (LD). Evaluation of these typing cells in pedigreed monkey families and in a random population indicated they identify antigens important in MLC stimulation which are different from but closely linked to the serologically defined transplantation antigens. These LD antigens appear to have a phenotype frequency of about 10% and 2% respectively. A panel of antisera raised to identify antigens expressed predominantly on B lymphocytes has been used to type 52 random unrelated monkeys and a computer assisted cluster analysis for identification of highly correlated antisera is underway.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To establish and maintain a colony of pedigreed rhesus monkey families for study of the genetics of transplantation and as an eventual source of donor-recipient pairs for transplant experiments.

This work is being conducted at Litton-Bionetics under contract N01-CB-64016. Dr. Bruce Maurer is the Prinicpal Investigator. Dr. Neefe is the Project Officer.

Methods Employed: Sera are raised by immunization with skin graft and peripheral blood lymphocytes of selected monkeys previously typed for RhLA major histocompatibility complex antigens and appropriately matched to related or unrelated donors. Sera are analyzed for serologically defined antigens by titration against known positive and negative monkeys, by microcytotoxicity typing of families and large random unrelated populations and by standard statistical analysis of patterns of reactivity observed. Selected sera are further analyzed for monospecificity by absorption and titration.

Monkeys are typed for MLC locus (LD) antigens by performing mixed lymphocyte cultures using as stimulators the two available monkeys homozygous at this locus. These monkeys are among the progeny of a single male and represent both of that male's LD antigens. In an alternative method developed elsewhere for human studies, lymphocytes are primed by previous exposure to respond in culture only to a particular antigen. This method potentially permits typing for each of the parental antigens in any family.

Monkeys are typed for antigens expressed predominantly on B lymphocytes after a procedure designed to enrich a population of peripheral blood lymphocytes for true immunoglobulin-bearing cells. Extensively washed lymphocytes are allowed to adhere to anti-Fab attached to plastic and, after washing, the B cells are competed off with excess free immunoglobulin. These cells are typed by the standard complement-dependent microcytotoxicity procedure. Breeding is designed to produce RhLA homozygotes, RhLA identical siblings, and to enlarge existing families of full siblings.

Major Findings: Analysis of new alloantisera has resulted in improved definition or new identification of 21 RhLA alloantigens, 13 in the A series and 8 in the B series. This represents an increase of three in the past year. All except four are defined by more than one serum. In most cases, sera defining an antigen have very similar frequencies and are very highly correlated in 2 by 2 Chi-square analysis in a random population of monkeys. About 80% of the gene product of the two loci can be identified.

Members of our team participated in a Nonhuman Histocompatibility Nomenclature Conference at the 1976 Congress of the Transplantation Society during which 12 antigens meeting certain standards of definition were given a new internationally accepted nomenclature analogous to that recently adopted for the human major histocompatibility complex HLA.

Evaluation of the RhLA homozygotes as stimulators in mixed lymphocyte culture revealed they can identify antigens coded by a locus (LD) closely linked to but not identical with the locus coding for the serologically defined RhLA antigens. Frequency of the two antigens detected by these homozygous typing cells is 10% and 2% respectively. Experiments to adapt the primed lymphocyte typing to the monkey are underway.

Fifty-two random unrelated monkeys have been typed with a panel of 57 sera previously identified as having possible preferential reactivity with B lymphocytes. Computer assisted statistical analysis is in progress.

Breeding has resulted in seven live births.

Significance to Biomedical Research and the Program of the Institute: Transplantation may be important to cancer therapy in several ways. For example, bone marrow transplantation may be used to "rescue" patients from otherwise fatal hemopoietic toxicity of intensive chemotherapy. Or, specific immunotherapy involving transplantation of lymphoid cells sensitized to tumor antigens may be useful in some cancers. In developing such techniques it may be useful to use as an experimental model an animal species, such as the monkey, which is closely related to man. Successful exploitation of such a model requires detailed knowledge of the major histocompatibility complex of the species. Acquisition of this knowledge is the aim of this project.

Proposed Course of Project: Major emphasis will be upon identification of MLC loci and upon development of antisera for these determinants. Efforts further to improve definition of the RhLA serologically defined antigens by raising and analyzing new sera and to expand the quantity and immunogenetic usefulness of the family members will continue.

Publications

Maurer, B. A. and Neefe, J. R.: Definition of 18 SD antigens and 2 LD antigens of the RhLA monkey major histocompatibility complexes. Transplant. Proc. 9: 579-583, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05016-08 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. J. Hodes	Head, Immunotherapy Section	IB NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients participating in an NCI immunotherapy protocol are being tested for cytotoxic humoral and cellular immunity against melanoma vaccine cells. Increases in both humoral and cellular immunity have been associated with immunotherapy but not with other control arms of the protocol.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this project has been to carry out in vitro tests for cytotoxic humoral and cellular immunity against melanoma vaccine cells. Patients being tested are associated with an NCI melanoma immunotherapy protocol.

Methods Employed:

1) Direct, Cell-Mediated Cytotoxicity: Cytotoxicity is measured by the release of ^{51}Cr from labelled target cells incubated overnight with immune cells. Target cells are prepared from vaccine melanoma cell lines maintained in tissue culture. Normal and immune lymphoid-attacking cells are prepared from human peripheral blood. Peripheral blood cells are purified by centrifugation with Ficoll-hypaque. Studies of multiply transfused humans have shown that cytotoxicity in this assay is specific for direct cell-mediated reactions and independent of conventional complement, humoral antibody, and nonspecific toxins.

2) Lymphocyte Dependent Antibody (LDA) Mediated Cytotoxicity. This cytotoxic pathway is also measured by a chromium release assay. After ^{51}Cr labelling, melanoma target cells are washed by centrifugation and then incubated for 1/2 hour with heat-inactivated serum or plasma containing LDA. Target cells are again washed and then incubated with nonsenstizied attacking lymphoid cells from normal donors for 4 hours.

3) K cell activity: This test is basically carried out as an LDA mediated cytotoxicity assay. Known LDA-containing, poly specific antisera from multi-transfused donors are used as sources of LDA, and peripheral blood lymphoid cells from melanoma patients are used as effector cells. By titering the dose of effector cells, K cell activity (i.e. which will elicit LDA mediated cytotoxicity) from patients cells can be compared with the counterpart from normal donors' cells.

4) Complement-Dependent, Antibody-Mediated Cytotoxicity. The complement assay is a two-stage, ^{51}Cr-microcytotoxicity test utilizing rabbit complement and vaccine melanoma cell lines.

Major Findings: As of December 1976, 81 melanoma patients have been tested for humoral and cellular immune activity against vaccine melanoma cell lines. 22 of 23 patients randomized to immunotherapy developed complement dependent antibody against one or more of the vaccine cell lines after initiation of immunotherapy. Each of the 20 patients tested in this group developed lymphocyte dependent antibody. Only 1 of 58 patients randomized to a control group showed increasing complement dependent antibody activity following therapy (BCG), and that patient had significant complement dependent antibody activity against melanoma vaccine cell lines prior to therapy. None of 53 normal donors or 41 melanoma patients in control groups had lymphocyte dependent antibody against vaccine cell lines.

Cell mediated cytotoxicity against a vaccine cell line was increased in 5 of 5 patients tested frequently during the first 4 months after initiation of immunotherapy. 14 of 18 such patients, tested less frequently, also showed increased levels of cellular immunity following immunotherapy. None of the patients randomized to control groups showed increased cellular activity. A final point of interest: K cell activity has not increased in patients receiving immunotherapy.

Significance to Biomedical Research and the Program of the Institute: *In vitro* cytotoxicity tests are being used to help assess the effectiveness of immunotherapy for melanoma patients. Although these tests may detect patient responsiveness to allogeneic transplantation antigens, they nevertheless serve the useful purpose of detecting nonresponsive patients or an ineffective vaccine. In addition the quantitative levels of cellular and humoral responses, albeit of an unknown specificity, may fall with tumor induced nonspecific immune suppression and thus will be a useful parameter to correlate with the clinical progression of disease.

Proposed Course of Project: Patients on the melanoma immunotherapy protocol will continue to be tested for humoral and cellular cytotoxic immune responses to the vaccine cell lines.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05017-07 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biological Significance of HLA Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	W. E. Bunney, M.D.	Chief	AP, NIMH
	E. S. Gershon, M.D.	Senior Investigator	AP, NIMH

COOPERATING UNITS (if any)
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SUMMARY OF WORK (200 words or less - underline keywords)

HLA antigens have been associated with a variety of diseases. Our studies concentrate on malignant diseases, disorders of immunity and psychoses. We are testing the hypothesis generated by our previous work that long-term survival in leukemia and lung cancer is due to the possession of certain HLA antigens. Other diseases studied include melanoma, acute schizophrenia and manic-depressed psychoses. It is too early to report on HLA and survival in leukemia and lung cancer, although at diagnosis there appears to be an abnormal distribution of HLA-A antigens (see text) in leukemia. HLA-A2 is elevated significantly ($p < .02$) in acute schizophrenia; there appears to be no HLA association with manic-depressed psychoses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Definition of the natural biological significance of certain human cell surface antigens is our objective. We have been studying two groups of antigens. The first is an antigen or set of closely related antigens revealed after neuraminidase treatment of intact cells. The second group is the well-known HLA antigen system. Nothing as yet is known of the biological significance of the first group of antigens. The latter group has importance for organ transplantation, but our emphasis is upon the relationship of HLA antigens to the natural function of the human organism.

We approached the significance of the neuraminidase revealed antigen(s) by studying the chemical nature of the antigen, its distribution on different cells and its naturally occurring autoantibody. We did not continue these studies this year and will not report on them any further.

We are seeking the significance of HLA antigens by investigating their frequency disturbances in different diseases. If such disturbances are found, and many have been, we can design further studies on the role of the antigen in the pathophysiology of diseases.

Methods Employed: HLA antigens are assayed by dye exclusion cytotoxicity tests. These are being conducted on contract by Drs. F. Ward and A. H. Johnson of Duke University (NO1-CB-52800).

Major Findings: Studies of the association of HLA antigens and disease continued in several areas. Most of the studies are in progress at present.

On the basis of previous work in acute leukemia and lung cancer, we hypothesized that the possession of certain HLA antigens conferred survival advantage to patients with these antigens. Testing this hypothesis requires the prospective typing of a large number of cancer patients. We are currently doing this. Through a collaboration with the Children's Cancer Study Group (previously Acute Leukemia Group A), we have just completed typing all their newly diagnosed acute leukemia cases. At over 600 patients typed, this is the largest series ever studied. We plan to ask two questions: a) are HLA antigen frequencies abnormal at diagnosis (implying a susceptibility/resistance factor to the development of the disease), and b) does survival correlate with possession of certain HLA antigens. Analysis of data from the first 372 patients indicated a significant, $p < .005$, perturbation of HLA-A locus antigen frequencies with HLA-A2 and Aw24 being elevated. No data is yet available on survival.

A similar study of Stage I lung cancer patients is being conducted with the NCI Lung Cancer Project at the Mayo Clinic. Over 150 patients have been typed. Data analysis has not been done yet.

We are continuing to type melanoma patients at NCI and the M. D. Anderson Hospital and to assay postimmunization (with a killed allogeneic melanoma cell vaccine) sera for anti-HLA activity. This data has not been completely analyzed yet.

We are continuing to assay HLA antigens in patients with well-documented acute schizophrenia, since there is some evidence for disordered immunity in this disease. In our series there continues to be a preponderance, significant at $p < .02$, of HLA-A2. More patients are being tested.

We are assaying HLA antigens in patients and family members with affective disorders, e.g., manic-depressive psychoses, since it is known that forms of this disease are genetic. We are looking for linkage between HLA and this disease. Thus far the data fails to suggest a linkage.

Finally, this contract serves to obtain the occasional HLA typing needed in the course of work of various investigators at NIH. This amounts to 10-15% of all HLA typing done.

Significance for Cancer Research and the Program of the Institute: Cell surface molecules are involved in important recognition phenomena controlling normal growth and development. Malignancies arise when this recognition mechanism fails. Thus the study of these molecules is of critical importance to understanding normal growth and development and malignancy. Fortunately many cell membrane structures are antigenic, allowing identification by standard immunologic techniques. It is our purpose to study selected plasma membrane antigens and their respective antibodies.

The cell surface histocompatibility antigens are a product of an extremely polymorphic genetic system which must have important survival value for the individual irrespective of its role in allograft rejection. For example, the possession of a given HLA specificity may confer, by mechanisms unknown, susceptibility (or resistance) to a particular disease. Therefore, one rational approach to assessing the natural biological significance of HLA antigen is the study of the association of disease with these antigens. If such associations are found, they have double significance. Closer medical observation of individuals possessing disease-associated HLA antigens may result in earlier diagnosis and better control of the disease. In addition, such knowledge may help us design experiments to probe the disease's basic pathophysiology.

Proposed Course of Project: A variety of patients with diseases, expecially malignancies and disorders of immunity, will be typed for HLA antigens. In particular, we will continue large scale prospective studies of the association of HLA antigens with survival in acute leukemia, lung cancer, and melanoma. Correlations will be sought, and experiments designed to probe the pathogenesis of the disease will be attempted. We will continue typing schizophrenics because of the suggested increase in HLA-A2 and the hypothesis that this may be an autoimmune disease.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05018-07 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Killer Lymphocyte Damage to Target Cell Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	C. K. Neels	Microbiologist	I NCI
	C. L. Stephens	IPA Investigator	I NCI
	R. B. Blumenthal	Senior Investigator	LTB NCI
	J. N. Weinstein	Expert	LTB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project previously established a model system to study the effects of killer lymphocytes on foreign cell membranes by using antibody coated lipid bilayers as target membrane for attack by normal human lymphocytes. This project is being extended in two ways. First, Dr. Stephens has set up the apparatus for intracellular electrical recording of target cells, so that the membrane events of immune lytic mechanisms can be studied directly. Second, antigenic liposomes have been prepared, and the release of fluorescent dye used to follow the action of antibody-mediated lymphocyte attack on these membranes. Preliminary results indicate that killing conditions do give rise to marker release from the liposomes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Electrical measurements on target cells will tell us if we can detect changes in membrane conductance of real cells, similar to those previously observed with synthetic membranes, and should give us further information on the cell's ability to repair membrane damage. The liposomes are being used because we can get information on the size of the membrane lesions by using different sized markers inside, and also can systematically vary the lipid composition to see what role the lipids play.

Methods Employed: The standard methods of intracellular electrical recording used by neurophysiologists have been adapted for the study of tumor target cells in culture. We have initially been using neuroblastoma cells as target cells for this part of the project. The liposomes have been made from synthetic phospholipids, cholesterol, and DNP-caproyl-phosphatidylethanolamine and filled with carboxyfluorescein. Rabbit anti-TNP antibodies bind to these liposomes. We have tried both sonicated, unilamellar small liposomes, and the multilamellar liposomes formed by vortexing. We have used two different geometries: liposomes in suspension bound to the Fc receptors of human lymphocytes in suspension, and liposomes bound to a surface being acted on by lymphocytes settled onto the surface.

Major Findings: No major findings have yet been made by electrical recording of the target cells because the apparatus is still not operating reliably, probably due to vibrations in the building. Our studies with liposomes have made substantial progress. Liposomes made of dioleoyl lecithin (DOL) cholesterol and DNP-cap PE, both multilamellar and unilamellar, bind to a subpopulation ($\sim$10%) of human lymphocytes in the presence, but not the absence of rabbit anti-TNP. This binding can be observed in the fluorescence microscope and by fluorometry by incubation of lymphocytes and liposomes in suspension. However, carboxyfluorescein from the bound liposomes is released more slowly than the carboxyfluorescein from control liposomes not bound to lymphocytes. If the multilamellar liposomes are immobilized on plastic surfaces, then coated with rabbit anti-TNP, a lymphocyte and antibody-dependent enhanced dye release has been observed in two out of two experiments with DOL.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors *in vivo*. Studies such as ours of the details of this process can be carried out *in vitro* and studied at the cellular and molecular level. Such studies are needed to complete our basic knowledge of the details of this important immunological defense process. Our studies have clearly shown that the target cell membrane is probably the primary target of killer lymphocyte attack, and the present studies have enabled us to clarify the nature of the membrane lesion by rigorous methods.

Proposed Course of Project: The major experiments using intracellular recording are still to be done. Membrane potential, resistance, and capacitance will be measured after immunological attack by antibody + C', ADCC or killer T lymphocyte micromanipulation of attacker lymphocytes will be utilized. The process of target cell destruction will be followed at the same time by phase microscopy.

Liposome marker release as a model for ADCC needs further confirmation and control experiments. If these experiments demonstrate that marker release occurs only under conditions of target cell destruction, we will systematically evaluate the effects of lipid composition, liposome size and relative rates of release of different sized markers.

Publications

Blumenthal, R., Weinstein, J. N., and Henkart, P.: Lipid model membrane studies on immune cytotoxic mechanisms. In Miller, M. W., and Shamoo, A. E. (Eds.): Proceedings of the 9th Rochester International Conference on Environmental Toxicity-Membrane Toxicity. New York, Plenum Press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05021-06 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Antigens Determined by the Major Histocompatibility Locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Head, Transplantation Biology Section	I NCI
OTHER:	T. H. Hansen	Postdoctoral Fellow	I NCI
	N. Shinohara	Visiting Associate	I NCI
	J. K. Lunney	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are being directed towards an understanding of the role of the products of the major histocompatibility complex in the cellular interactions of the immune response. Projects can be divided into three major categories: 1) Characterization of Major Histocompatibility Antigens: Congenic resistant strains of mice have been reciprocally immunized to produce antisera detecting the products of limited regions of the MHC. Antigens determined by genes in the Ir region termed Ia antigens have been detected and have been shown to provide a major source of stimulation of the MLC reaction; 2) Characterization of the Response to Major Histocompatibility Antigens: The effect of genetic background on the ability to respond to particular Ia and H-2 antigens is being assessed; and 3) Characterization of the Receptor Site for Histocompatibility Antigens: Anti-idiotypic antibodies are being produced and their effects on in vitro and in vivo parameters of graft rejection are being assessed.

Project Description

Objectives: 1) To produce antibodies against defined regions of the major histocompatibility complex. 2) To characterize the reactions of these antibodies with the cell surface of lymphocytes, and to determine the nature of the cells bearing individual antigens. 3) To attempt to produce anti-idiotypic antibodies against the receptors on these antibodies which detect cell surface histocompatibility antigens.

Methods Employed: Congenic resistant strains of mice differing only at their major histocompatibility loci have been reciprocally immunized in order to produce antibodies of known, restricted specificity. The antibodies have been characterized by assays of complement-mediated lymphocytotoxicity and by fluorescence microscopy using a fluoresceinated rabbit antimouse immunoglobulin as a developing agent.

The presence of unexpected specificities in these sera reacting with subpopulations of lymphoid cells has led to the development of methods of distinguishing subpopulation reactions. Additivity studies for cytotoxicity reactions and fluorescent staining have been performed with these reagents in combination with reagents of known specificity for B or T subpopulations of lymphoid cells. Also, reactions with fractionated lymphoid cell populations have been performed. T-cell derived tumors have been used for the absorption of the congenic antisera in order to reveal further specificities for antigens expressed preferentially on B cells. In addition, use has been made of congenic resistant animals bearing recombinant H-2 alleles in order to determine the genetic localization of many of the new antigens.

Antibodies against individual antigenic specificities have been isolated by adsorption onto lymphoid cells bearing those specificities, and the antibody-coated cells have then been injected back into syngeneic animals in order to immunize against the "receptor" portions of the antibody molecules. Sera have been collected from these animals after multiple immunizations and are being characterized for the presence of anibodies capable of reacting with the appropriate antihistocompatibility antigen receptors of antibodies and cells. Tests include inhibition of cytotoxicity, binding to solid-phase immunoabsorbents, passive hemagglutination, and *in vitro* and *in vivo* blocking of the immune response to tissue antigens.

Similar protocols are also being applied to antibodies raised in inbred rats differing at multiple histocompatibility loci, and to cross-species systems.

Major Findings: 1) Several of the antisera produced in congenic resistant mice have been shown to contain specificities additional to those expected in these strain combinations. These specificities have been shown to be determined by genes mapping within the Ir region of the murine major histocompatibility locus, and to be expressed preferentially on B cells. Similar antigenic specificities have simultaneously been described by other laboratories, and the name Ia (Ir associated) antigens has been adopted to

describe this new class of cell-surface antigens. Anti-Ia antibodies have been demonstrated in several H-2 antisera by absorption with T cell tumors.

2) The discovery of one of these Ia specificities on the B cells of a congenic recombinant mouse differing from another mouse only in the Ir region has been found to correlate with the previously described "one-way" mixed lymphocyte culture (MLC) reaction in this strain combination. This has led to the hypothesis that these Ia antigens may be associated with or responsible for the MLC reaction. This hypothesis has been confirmed experimentally using genetically defined MLC combinations and separated cells. Antibodies to distinct Ia antigens have been shown to specifically inhibit the MLC reaction at the level of apparent block of the stimulator cell.

3) Anti-Ia antibodies have been found to specifically block the Fc receptor of B lymphocytes. In addition one other genetic locus not linked to H-2 has been found to determine antigen(s) associated with the Fc receptor.

4) Anti-idiotypic reactions against the anti-H-2 antibodies have so far been demonstrable only in the presence of anti-allotype reactions and have been extremely weak.

5) An antibody response to a previously unrecognized H-2 antigen has been found and has been determined to be genetically controlled by a non-H-2 linked locus.

6) Antibodies to Ia like antigens of the rat have been detected in anti-AgB antisera by absorption of the antisera with rat platelets. By polyacrylamide gel electrophoresis antigens precipitated by these antisera have been shown to be of the same size as mouse Ia antigens.

7) Cytotoxicity tests and absorption analyses on a congenic anti-Ia antiserum have suggested the presence of another subregion within the Ir region of the H-2 complex. This subregion has been given the name I-E

8) Analysis of anti-histocompatibility antisera by gel electrophoresis of labelled cell surface antigens has demonstrated a new H-2 product determined by a previously unrecognized H-2 region closely linked to H-2D, termed D'.

Significance to Biomedical Research and the Program of the Institute: 1) The Ia antigens appear to provide a new cell-surface marker for the B subpopulation of lymphocytes. In addition, the localization of the genes determining these antigens to the Ir region may indicate that these B cell surface structures are involved in the T cell-B cell collaboration necessary for many immune responses. If so, these antigens may provide markers for dissecting the mechanism of that collaboration.

2) The specificity of the receptor for histocompatibility antigens should reside in the variable portion of the heavy and light chains of the relevant antibody molecules. Thus, anti-idiotypic antibodies against such receptors might be expected to distinguish those cells capable of reacting against

individual histocompatibility antigens. Such antibodies, if obtained, could thus provide a new approach to modification of the immune response to cell surface antigens.

3) Understanding of the genetic control of response to H-2 antigens may make possible the modification of these responses in order to produce tolerance.

Proposed Course of Project: 1) Other antisera raised between congenic resistant strains of mice differing by known regions of the major histocompatibility complex will be screened for additional Ia specificities. The cellular distribution of these specificities will be determined (i.e., T cells vs B cells), and lymphoid tumors of known cellular origin will be used to absorb differential activities.

2) Models for the functional properties of these antigens which may explain their genetic localization to the Ir region will be further explored. These will include MLC reactivities, cellular cooperation models and cellular cytotoxicity models.

3) The relationship between Ia antigens and the Fc receptor will be pursued. Other non-H-2 antigens also associated with the Fc receptor will be investigated, as well as the Fc receptor of other cell populations such as macrophages and T cells.

4) Attempts at immunization against the receptors of anti-H-2 antibodies and cellular receptors for alloantigens will be carried out. Xenogenic immunization will be attempted.

5) Extensive testing as outlined above will be required in order to determine whether or not anti-idiotypic antibodies have been produced in any of our immunization protocols. If we can demonstrate the presence of such antibodies serologically, we will then attempt to purify them by selective immunoabsorption on columns bearing the appropriate immunoglobulins. The effects of these antibodies on *in vivo* cellular and humoral responses to tissue grafts will then be tested.

6) Additional strain distribution studies will be performed in order to determine whether the new subregion of H-2 (D') has other alleles which can be detected in other haplotypes.

Publications

Schwartz, R. H., Fathman, C. G., and Sachs, D. H.: Inhibition of stimulation in murine mixed lymphocyte cultures with an alloantiserum directed against a shared Ia determinant. *J. Immunol.* 116: 929-935, 1976.

Cullen, S. E., David, C., Cone, J., and Sachs, D. H.: Evidence for more than one Ia antigenic specificity on molecules determined by the I-A subregion of the mouse major histocompatibility complex. *J. Immunol.* 116: 549-553, 1976.

Sachs, D. H., Cone, J., and Humphrey, G. W.: The humoral response to an Ia antigen and skin graft rejection: Mechanistic possibilities. Transplant. Proc. 8: 413-416, 1976.

Sachs, D. H., Schwartz, R. H., and Fathman, C. G.: The role of Ia antigens in MLC stimulation. In Katz, D. H. and Benacerraf, B. (Eds.): Proceedings of The Role of Products of the Histocompatibility Gene Complex in Immune Responses. New York, Academic Press, 1976, pp. 159-165.

Schwartz, R. H., Sachs, D. H., David, J., and Paul, W. E.: T lymphocyte enriched murine peritoneal exudate cells. III. Inhibition of antigen-induced T lymphocyte proliferation with anti-Ia antisera. J. Immunol. 117: 531-540, 1976.

Dickler, H. B., Arbeit, R. D., and Sachs, D. H.: The association between Fc receptors and certain lymphocyte alloantigens. In Seligmann, M. (Ed.): Membrane Receptors of Lymphocytes. Amsterdam, North-Holland Pub. Co., 1975, pp. 259-266.

Dickler, H. B., Arbeit, R. D., Henkart, P. A., and Sachs, D. H.: Association between Ia antigens and the Fc receptors of certain T lymphocytes. J. Exp. Med. 144: 282-287, 1976.

Hansen, T., Cullen, S., and Sachs, D. H.: Immunochemical evidence for an additional H-2 region closely linked to H-2D. J. Exp. Med. 145: 438-442, 1977.

Schwartz, R. H., Dickler, H. B., Sachs, D. H., and Schwartz, B. D.: Studies of Ia antigens on murine peritoneal macrophage. Scand. J. Immunol. 5: 731-743, 1976.

Sachs, D.: Evidence for an I-E subregion. Proceedings of Third Ir Gene Workshop, December 1976, in press.

Hansen, T., Cullen, S., Shinohara, N., Schurko, E., and Sachs, D. H.: Genetic control of the humoral response to an H-2 public specificity. J. Immunol., in press.

Shinohara, N., and Sachs, D. H.: Genetic control of the immune response to H-2.32. In Proceedings of ICN-UCLA Symposia - Regulation of the Immune System: Genes and The Cells in Which They Function, in press.

Ahmann, G. B., Sachs, D. H., and Hodes, R. J.: Genetic analysis of Ia determinants expressed on Con A reactive cells. In Proceedings of ICN-UCLA Symposia - Regulation of the Immune System: Genes and The Cells in Which They Function, in press.

Hansen, T., Cullen, S., Melvold, R., Kohn, H., Flaherty, I., and Sachs, D.: A mutation in an H-2 associated histocompatibility gene other than H-2K or H-2D. J. Exp. Med., in press.

Shinohara, N., Cullen, S. E., and Sachs, D. H.: Ag-B-linked analogue of Ia antigens in the rat. J. Immunol., June 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05023-06 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Transplantation Antigens of Swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Head, Transplantation Biology Section	I NCI
OTHER:	S. A. Rosenberg	Chief, Surgery Branch	S NCI
	R. L. Kirkman	Clinical Associate	I NCI
	J. K. Lunney	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

NIH Animal Center, Poolesville, Maryland
W. Flye, Walter Reed Army Institute of Research

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A breeding program has been carried out starting with two miniature pigs from different sources and selecting offspring according to tissue typing procedures aimed at defining the major histocompatibility complex of this species. By this procedure three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. MLC reactions, serologic typing, and skin graft survival times all indicate that these animals are homozygous for a major histocompatibility locus analogous to HL-A of man or H-2 of the mouse.

Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for tissue typing and transplantation; 2) Purification and characterization of the major histocompatibility antigens of this species; and 3) Assessment of the immunologic parameters involved in tolerance to liver allografts in this species. Inbreeding in brother-sister fashion within each of these herds will be continued with the ultimate goal of establishing truly inbred animals if possible.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop and maintain three strains of miniature swine, each homozygous for a different set of histocompatibility antigens at the major histocompatibility locus (MSL-A). The animals are used for in vivo experiments in organ and tissue transplantation and as a source of large numbers of cells from which cell surface antigens are isolated, purified, and characterized chemically.

Methods Employed: Unlike classical inbreeding schemes which require about twenty generations to produce homozygosity, the approach being used with these swine involves selective breeding on the basis of histocompatibility typing of parents and offspring.

Four males and four females were purchased from commercial sources, and mating pairs were selected to be as varied as possible in order to assure the selection of different histocompatibility genes. Skin grafts were exchanged between the members of each pair and sera were obtained from the animals two weeks after the rejection of the grafts. The sera were tested for cytotoxic antibodies by lymphocytotoxic typing, and pairs which produced strong cytotoxic antibodies were bred.

Offspring from each breeding were tested serologically to determine which histocompatibility antigens had been inherited from the parents, and offspring which could be shown to possess the same antigen combinations were selected for further breeding. This process has now been repeated for six consecutive generations.

Transplantation of tissues in these animals has been performed in the large animal facility of the Surgery Branch in Building 14. Methods have been developed for the transplantation of split thickness skin grafts from the ear to the dorsal thorax. Allografts are always placed side by side with autografts as a control. The use of ear skin permits very accurate assessment of viability of the grafts and determination of rejection times. Surgical techniques for vascularized grafts (kidney and liver) are being developed.

Chemical purification studies on swine transplantation antigens are being performed using detergent solubilized cell surface preparations from spleen and lymph node cells. Initial studies involved radiolabelling of the surface antigens with tritiated leucine by published methods, and the assessment of molecular weight of those components which react with alloantisera by the use of SDS polyacrylamide gels. Methods are currently being developed for handling larger quantities of histocompatibility antigens without the use of radiolabelled amino acids.

Major Findings: 1) Naturally occurring anti-red cell antibodies of swine are cytotoxic to lymphocytes (unlike their human counterparts) and must be absorbed from sera with packed red cells before leukocyte typing is performed.

2) Skin grafts between appropriate third and fourth generation animals were rejected in 8-14 days, and the rejection was accompanied by the production of high-titered lymphocytotoxic antibodies. Boosts with lymphocytes have been carried out in several related combinations and have yielded large quantities of high-titered antiserum.

3) Breeding of further generations has continued to be successful.

4) Typing of the fourth generation offspring yielded three breeding pairs of animals homozygous for serologically determined transplantation antigens. We therefore now have the capacity to breed three different herds of swine, each homozygous for a different SL-A antigen.

5) MLC assays of parents and offspring showed a close correlation between this parameter of histocompatibility and the serologically determined transplantation antigens. Since this type of correlation has previously been observed in studies of man (HL-A), monkey (RhL-A), mouse (H-2), rat (AgB) and dog (DL-A), this finding indicates that we are selectively breeding for homozygosity at a major histocompatibility complex (MHC) homologous to that of other mammalian species.

6) Skin graft survival has been found to be prolonged within each of the homozygous herds. The mean survival time for grafts within homogyzous herds was $11.8 \pm .89$ days, while the mean survival time of skin grafts between animals of the three different herds was 7.0 ± 0.36 days.

7) Renal allografts within the DD homogyzous herd appeared to survive indefinitely despite minor histocompatibility antigen differences. Some rejection occurred following allografts within the other two herds, but too few experiments have been done to be sure that this is rejection and not technical failure.

8) Alloantisera between the three herds have been analyzed by gel electrophoresis using detergent solubilized cell surface antigen preparations. Peaks were obtained at 45,000 molecular weight corresponding to the mouse H-2 antigen analog. Peaks at 35,000 and 28,000 molecular weights corresponding to mouse Ia antigens were also observed. Both by size criteria and by genetic criteria these antigens thus appear to be the precise homologs of H-2 and Ia antigens of the mouse.

Significance to Biomedical Research and the Program of the Institute: One of the major problems in the study of cell surface antigens of human beings is lack of control of genetic constitution. The use of mice and rats as experimental models avoids this problem, but creates two new ones: 1) physiologically and anatomically these animals are often so different from human beings as to make comparisons and applications of findings difficult. 2) The size of these rodents makes it extremely difficult to obtain sufficient cells and tissues to permit quantitative chemical characterization of relevant cell surface antigens.

For both of these reasons it is desirable to have animals of size comparable to human beings, whose genetic constitution with respect to histocompatibility can be controlled. The miniature pig, which attains an adult weight of about 200 lbs, is ideal for this purpose, and to date the experimental breeding pattern outlined above appears to be working well.

Proposed Course of Project: The breeding plan and typing will be continued. Lines will be established and separated, so that herds of any required size in each line can be produced.

Experiments in collaboration with the Surgery Branch will be continued in order to: 1) characterize the immune response to transplantation of skin, kidney, and liver in pigs across defined histocompatibility differences, and 2) to determine the basis of liver allograft tolerance which has previously been reported in pigs.

Studies of the MSL-A antigens at the biochemical level will be continued. These will center first on isolation and purification, and then on chemical and immunologic characterization.

Publications

Sachs, D. H., Leight, G., Cone, J., Schwartz, S., Stuart, L., and Rosenberg, S.: Transplantation in miniature swine. I. Fixation of the major histocompatibility complex. Transplantation 22: 559-567, 1976.

Leight, G. S., Sachs, D. H., Williams, G. M., and Rosenberg, S. A.: Transplantation in miniature swine. Transplant. Proc. 9: 575-577, 1977.

Leight, G. S., Sachs, D. H., and Rosenberg, S. A.: Transplantation in miniature swine II. In vitro parameters of histocompatibility in MSLA homozygous minipigs. Transplantation 23: 271-276, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 05033-06 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. D. Terry	Assoc. Dir. for Immunology	I	NCI
Other:	R. J. Hodes	Head, Immunotherapy Section	I	NCI
	S. A. Rosenberg	Chief, Surgery Branch	SURG	NCI
	R. I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

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Immunology Branch

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INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of more than 100 patients have entered the trial, but it is too early to evaluate the results.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designated IB-2) was initiated in August 1975. This protocol will evaluate the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients are stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they are randomized to receive no further therapy (this is standard treatment for these patients) or chemotherapy with methyl-CCNU or immunotherapy with BCG, or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines. These cell lines were a gift from Dr. Donald Morton, UCLA, and are grown in PPLO-free conditions at Litton-Bionetics under the supervision of Dr. Edwin Matthews. Cells are treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

All patients are worked up by the Immunology Branch or the Surgery Branch. Following randomization, they are assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods are drawn during pre-randomization work-up and subsequent to randomization assays of cell-mediated and humoral immunity are performed.

Major Findings: As of May 26, 1977, 109 patients have been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	26	24	30	29
Recurrences	10	7	12	9
Deaths	4	5	6	6

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see CB-05016). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course: The IB-2 protocol will be continued.

Publications

Rosenberg, S.A. and Terry, W.D.: Passive immunotherapy of cancer in animals and man. Adv. Cancer Res. 25: 323-388, 1977.

Terry, W.D.: Present status and future directions for cancer immunotherapy. Gann Monogr. Cancer Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05034-05 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization and Isolation of Human Lymphocyte Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler Senior Investigator I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to characterize and isolate human lymphocyte subpopulations and evaluate the function of these populations in the immune response of both normal and diseased individuals. Recent work under this project has shown that anti-HL-A alloantisera contain antibodies against antigens which are found primarily on B lymphocytes and which are associated with B lymphocyte Fc receptors. In addition it was found that the Fc receptors of B lymphocytes and UL cells differ both in their avidity for Ig complexes and in the fact that the Fc receptors of UL cells do not appear to be associated with "Ia-like" antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To characterize and isolate human lymphocyte subpopulations and evaluate the function of such populations in the immune response in both normal and diseased individuals.

Methods Employed: Lymphocytes from normal human donors were reacted with alloantisera and then evaluated for the presence of bound antibody and function of Fc receptors by fluorescent techniques. Alloantisera were fractionated by absorption with and elution from platelets. Antigen-antibody complexes and double marker fluorescence techniques were used to evaluate the avidity of lymphocyte subpopulations for Ig complexes. A system for in vitro PFC responses to soluble antigens is being developed.

Major Findings: Certain anti-HL-A alloantisera contained antibodies which inhibited the binding of complexed Ig to B lymphocyte Fc receptors. After absorption with platelets the alloantisera lost HL-A reactivity but retained the ability to react with B lymphocytes and inhibit Fc receptor function. Conversely, the HL-A antibodies eluted from platelets reacted with all lymphocytes but did not inhibit binding to Fc receptors. These data suggest that human B lymphocytes bear "Ia (Immune Response gene associated) like" antigens and that these antigens are associated with B lymphocyte Fc receptors.

B lymphocytes and UL cells were shown to have Fc receptors which were distinct. UL cells Fc receptors had a much higher avidity for Ig complexes and were not associated with "Ia-like" antigens. These differences may be of functional importance.

Significance to Biomedical Research and the Program of the Institute: Characterization and isolation of lymphocyte subpopulations is of fundamental importance for an understanding of the nature of the immune response.

By analogy to the murine system, identification of human alloantigens associated with Fc receptors and primarily expressed on B cells may be important for understanding immunoregulation and interactions between immunocompetent cells, and may help characterize the genes responsible for such regulation.

Proposed Course of Project: If in vitro antibody responses to soluble antigens can be obtained, this system would be used to evaluate a) Ir gene function in man; b) role of various cell populations in tha antibody response in man; and c) role of Ir genes in human disease.

Publications

Dickler, H. B.: Lymphocyte binding of aggregated immunoglobulin. Scand. J. Immunol. Suppl. 5: 91-97, 1976.

Dickler, H. B.: Lymphocyte receptors for immunoglobulin. Adv. Immunol. 24: 167-214, 1976.

Dickler, H. B.: Studies of the association between Ia antigens and Fc receptors. In Siskind, G. W., Christian, C. L., and Litwin, S. D. (Eds.): Clinical Evaluation of Immune Function in Man. New York, Grune and Stratton, 1976, pp. 13-26.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05035-05 I

PERIOD COVERED: July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of the Lymphocyte Receptor for Complexed Immunoglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	IB NCI
OTHER:	D. H. Sachs	Head, Transplantation Biology Section	IB NCI
	D. L. Nelson	Investigator	MB NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Immunology Branch

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.25	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to characterize lymphocyte Fc receptors genetically and functionally. Recent findings indicate that the Fc receptors of B cells and T cells but not UL cells nor macrophages are associated with Ia antigens. In addition, a subpopulation of UL cells, the effector cells in antibody-dependent cell-mediated cytotoxicity was shown not to bear Ia antigens. In the case of the B cell, the Fc receptors and Ia antigens appear to be distinct molecules, and the association appears to be ligand induced. There is also a single non-H-2 locus which determines antigens associated with the Fc receptor. In one haplotype, this locus has been shown to be linked to the M locus. These findings indicate that Fc receptors may play a key role in immunoregulation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) To evaluate the genotypic and phenotypic expression of the Fc receptor; (2) to evaluate the role of this receptor in the immune response.

Methods Employed: Heat-aggregated immunoglobulin and/or antigen-antibody complexes are fluorochrome or radioactive isotope labelled, and then allowed to interact with isolated lymphocytes or macrophages from animals. Binding is assayed by U.V. and phase microscopy, the Fluorescence Activated Cell Sorter (FACS), or by isotope counting. The interaction is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both lymphocytes and complexes. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Functionally, the effect of Ig complexes on the humoral immune response (plaque-forming cell assays) and antibody dependent cell-mediated cytotoxicity (ADCC) are analyzed.

Major Findings: The Fc receptors of murine B lymphocytes are associated with Ia antigens in that anti-Ia antibodies specifically inhibit binding of Ig complexes to Fc receptors. However, the two moieties appear to be distinct molecules in that capping most Fc receptors does not appear to alter the distribution of Ia antigens. In addition, quantitative analysis of Ia antigens using the FACS before and after binding of complexes to Fc receptors and/or capping Fc receptors did not alter the amount of Ia antigens detected. Thus, since the two moieties appear distinct, and since inhibition of detection of Ia antigens was not detected upon binding of complexes to Fc receptors, the association does not appear to be steric in nature. The most plausible explanation for the inhibition of detection of Fc receptors by anti-Ia antibodies is that the binding of a ligand to Ia induces an association with Fc receptors.

Antigens determined by at least one non-H-2 locus are also associated with B cell Fc receptors. The antigens determined by this locus appear to be mainly expressed on B lymphocytes and appear to be distinct from Fc receptors on the basis of capping experiments. The locus itself is not linked to the major histocompatibility complex, the Ig allotype locus and is not sex-linked. It is, however, closely linked to the M locus.

A portion of T lymphocytes also have Fc receptors but these appear to be of either lower affinity or are present in fewer numbers per cell than the Fc receptors of B lymphocytes. They also appear to be associated with Ia antigens on certain T lymphocytes.

The Fc receptors of macrophages and UL cells do not appear to be associated with Ia antigens. In addition, most UL cells including those that mediate ADCC lack Ia antigens.

Significance to Biomedical Research and the Program of the Institute: The receptor for complexed antibody has been shown to play a key role in antibody-dependent cell-mediated cytotoxicity. This immunologic mechanism may play an important part in host response to tumors. The Fc receptor/Ia antigen association is hypothesized to be important in regulation of the humoral immune response and collaborations between immunocompetent cells.

Proposed Course of Project: (1) Studies of the Ia-Fc relationship by capping of Ia antigens and other cell surface markers followed by evaluation of Fc receptors. (2) Use of pharmocologic agents and techniques evaluating mobility of cell surface proteins to evaluate the relationship of Fc receptors to other surface molecules. (3) Analysis of the requirements for alteration or aggregation of Ig which allow binding to Fc receptors. (4) Analysis of the role of Fc receptors in regulation of the immune response using various functional assays.

Publications

Dickler, H. B.: Lymphocyte binding of aggregated immunoglobulin. Scand. J. Immunol. Suppl. 5: 91-97, 1976.

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Schwartz, R. H., Dickler, H. B., Sachs, D. H., and Schwartz, B. D.: Studies of Ia antigens on murine peritoneal macrophage. Scand. J. Immunol. 5: 731-743, 1976.

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Dickler, H. B., Arbeit, R., Cowing, C., Henkart, P., Kubicek, M., Nelson, D., and Sachs, D. H.: Studies of the relationship between Ia antigens and Fc receptors on various cell populations. Immunopathology, in press.

Dickler, H. B., Kubicek, M. T., Arbeit, R. D., and Sharrow, S. L.: The association between Ia antigens and Fc receptors on murine B lymphocytes is probably ligand-induced. In Proceedings of the Third Ir Gene Workshop, in press.

Dickler, H., Kubicek, M., Arbeit, R., and Sharrow, S. O.: Studies on the nature of the relationship between Ia antigens and Fc receptors on murine B lymphocytes. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05036-05 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Staphylococcal Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Head, Transplantation Biology Section	I NCI
OTHER:	D. S. Pisetsky	Clinical Associate	I NCI
	J. A. Berzofsky	Expert	M NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch, DCBD, NCI

SECTION
Transplantation Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies directed against idiotypic determinants on anti-staphylococcal nuclease antibodies from different mouse strains have been produced in rats and can be detected by the inhibition of antibody-mediated inactivation of nuclease. These anti-idiotypes have been shown to be specific for the combining site (variable regions) of anti-nuclease antibodies since extensive absorption with normal mouse immunoglobulins fails to diminish their activity, and since antibodies to other sites on the Ig molecule do not inhibit inactivation. As nuclease is an antigen, the response to which is under control of H-2 linked immune response genes, it was of interest to determine the relationship between the idiotype and other genetic loci. By screening a variety of strains and offspring from appropriate matings between strains for the presence of idiotypes and other markers it has been shown that idiotype expression is linked to the heavy chain allotype marker and not to the immune response gene locus. The relationship of the idiotype to the problem of T-cell recognition of antigen is being investigated.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The antibody response to staphylococcal nuclease (NASE) is under genetic control by gene(s) located in the Ir region of the mouse major histocompatibility complex (H-2). Since the antibodies produced to certain regions of this antigen have been previously shown to demonstrate restricted heterogeneity, we wish to determine whether or not the relevant Ir gene determines which variable region immunoglobulin products are produced. If the Ir gene is found to exert quantitative but not qualitative control over idiotypic antibody production, then a search for a distinct "T cell receptor" for nuclease (i.e., other than known IgV regions) must be anticipated.

Methods Employed:

1) Antibodies to NASE have been prepared in groups of mice of a variety of strains (both high and low responders) differing in H-2 type or allotype or both. Certain of these have been purified by affinity chromatography. Inbred Lewis rats have been immunized with the purified anti-NASE antibodies obtained from immune ascites from two A/J mice and SJL mice after immunization with NASE. Immunoabsorptions with normal immunoglobulins from the same strains have been used in order to determine whether or not antibodies reactive with the variable region (i.e., anti-idiotypic antibodies) have been produced.

2) Anti-idiotypic reactions have been quantitated by the inhibition of antibody-mediated enzyme inhibition. Such reactivities have been screened against anti-NASE antibody populations from the other strains of mice in order to determine the possible genetic linkage of idiotype to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idiotype expression in order to determine linkage of idiotype to other genetic markers.

4) Antibodies to nuclease have been separated on affinity columns into subpopulations directed against different determinants of nuclease. The reactions of anti-idiotypic antisera with these subpopulations have been characterized in order to determine new idiotypic markers.

5) Antibodies to individual determinants of nuclease have been measured in antisera from high and low responder strains. A radioimmunoassay using labelled polypeptide fragments of nuclease has been developed for this purpose.

Major Findings: By two separate criteria, anti-idiotype antibodies have been obtained. 1) After exhaustive absorption with whole normal A/J ascites or repeated passages over affinity chromatography columns to which normal A/J ascites was bound, antibodies remained which reacted with A/J αNASE but not with B10.A antibody. Prior to absorption these rat antibodies contained precipitating antibodies to either type (A/J or C57BL10) of antibody but no precipitating antibodies remained following absorption. 2) The rat anti-A/J

αNASE antibodies prior to and following absorption reacted with the combining site of anti-NASE antibodies as evidenced by their ability to inhibit the anti-NASE antibody inactivation of NASE in enzymatic assay.

Among (B10.AxA/J)xB10.A backcross animals the A/J αNASE idiotype was found to be linked ($p<.005$) to heavy chain allotype. However, a large recombination frequency was found (7-10 percent). The use of a very sensitive assay for allotype indicated that this recombination frequency was not the result of faulty allotypic typing. Also, progeny testing of the putative recombinant animals showed approximately 50 percent of the offspring to also have recombinant phenotypes, further indicating that these were true recombinant animals.

Following multiple boosts with antigen congenic high and low responder animals (B10.A and B10 respectively) were both found to make comparable levels of antibody. However, the proportion of antibody against the carboxy terminal third of the nuclease molecule was very different between these two strains. This indicates that H-2 linked Ir gene control must exert an effect at the B cell level as well as at the T cell level in cell interactions of the immune response.

Significance to Biomedical Research and the Program of the Institute: In order to understand the mechanisms involved in immune responses it is important to have well characterized model antigens which can be studied. Many of the antigens being studied in this laboratory are difficult to obtain in pure form (such as transplantation antigens and tumor antigens) and it is therefore important to have a well characterized protein model on which initial experiments can be performed. In choosing models both for mechanistic studies and for studies in manipulation of the immune response, staphylococcal nuclease is a particularly attractive protein. The immune response to this antigen has previously been shown in this laboratory to be under genetic control by an H-2 linked Ir gene.

The development of anti-idiotypic reagents directed toward antibodies under the control of an H-2 linked Ir gene provides another handle to study the mechanism of Ir gene function. They may help to determine whether the specificity of T cell recognition of antigens depends on similar variable region gene products to those responsible for B cell or antibody specificity. They may also lead to possible methods of controlling Ir gene expression.

Proposed Course of Project: There is suggestive evidence from other workers that the T cell antigen receptor shares the same idiotype as an antibody directed against that antigen. To study such a role for the idiotype in the generation of antinuclease antibodies, the effect of the anti-idiotype on the production of antinuclease antibodies will be investigated. As the stimulation as well as the abolition of antibody production are both possible expressions of anti-idiotype interaction with immune cell receptors, studies will be designed to assess both. An alternate approach, the effect of anti-idiotype antibodies on the generation in vitro of antigen specific T cell

factors, will be explored. If these two approaches fail to provide evidence for the role of the idiotype on T cells, an attempt will be made to immunize with immune T cells rather than antibody in order to determine whether another "idiotype" can be distinguished for the T cell receptor.

The anti-idiotype preparations currently available are heterogeneous with specificities directed against a variety of antinuclease antibodies. To define more precisely the specificities represented, antibodies against subregions of the nuclease molecule will be prepared and the interaction of the anti-idiotype with these fractionated antibodies studied. It is hoped that these fractionated preparations will provide more sensitive reagents in the study of the genetic basis for variable regions of antinuclease antibodies as well as possible study of the detailed structure of the nuclease receptor on T cells.

Each of the recombinant animals which have been found by assessment of idiotype in backcross progeny, will be established as a new line. A variety of other variable region markers will then be tested in these animals, in an attempt to develop a linear map of the variable region genes.

Further study of backcross progeny in several different strain combinations will be performed. This should help to determine recombination frequencies more accurately, and should also help to provide a better linear map of the variable region. Attempts to determine the basis for sharing of idiotypic determinants between strains will be made by the examination of closely related strains and sublines. This may shed light on the mechanisms for the generation of diversity of antibody specificities.

Publications

Fathman, C. G., and Sachs, D. H.: Genetic control of the immune response to nuclease II. Detection of idiotypic determinants by the inhibition of antibody mediated nuclease inactivation. J. Immunol. 116: 959-964, 1976.

Berzofsky, J. A., Schechter, A. N., Shearer, G. M., and Sachs, D. H.: Genetic control of the immune response to staphylococcal nuclease. III. Time course and correlation between the immune response to native nuclease and the response to its polypeptide fragments. J. Exp. Med. 145: 111-122, 1977.

Berzofsky, J. A., Schechter, A. N., Shearer, G. M., and Sachs, D. H.: Genetic control of the immune response to staphylococcal nuclease. IV. H-2 linked control of the relative proportions of antibodies produced to different determinants of native nuclease. J. Exp. Med. 145: 123-135, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05038-05 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to TNP-Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	A.-M. Schmitt-Verhulst	Expert	I NCI
	C. M. Pettinelli	Visiting Fellow	I NCI
	P. A. Henkart	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.5	1.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

T-cell-mediated lympholysis was generated in vitro against trinitrophenyl-(TNP-) modified syngeneic murine spleen cells. The cytotoxic effectors generated are specific for both the modifying agent and for self major histocompatibility complex (MHC) products mapping in the K or D regions of the H-2 complex. Specificity of the effectors was such that they could not lyse H-2-matched targets in which the TNP group was presented on the targets by insertion of a TNP-fatty acid-dextran conjugate into the cell membrane. However, effectors could be generated by culturing spleen cells with TNP coupled to soluble proteins. These effectors were also restricted to lyse TNP-modified K or D matched targets. Cultures primed in vitro with TNP-modified syngeneic cells generated secondary effectors only if restimulated with TNP-modified cells sharing K or D with the primary stimulators. Secondary proliferative responses were induced only with TNP-modified cells sharing K, D, or I regions with the primary stimulators. The ability of mouse spleen cells to generate effectors against TNP-H-2D^d was shown to be controlled by at least two H-2 linked immune response genes--one mapping between the I-A and I-J subregions and one to the left of the I region of the H-2 complex.

Project Description

Objectives: The objective of this project was to investigate the immunogenetic parameters associated with the in vitro generation of T-cell-mediated immunity of murine spleen cells to syngeneic lymphoid cells modified with the TNP group. More explicitly this project was designed to: (a) further elucidate the fine specificity (i.e., the contribution of the TNP moiety to the specificity) of the cytotoxic effector cells for TNP-modified self reactions; (b) determine whether in vitro secondary cytotoxic responses could be generated against TNP-modified syngeneic cells, and if so, whether restimulation was restricted to H-2 products coded for by K and D region genes; (c) establish whether a proliferative response could be obtained after secondary stimulation, and if so, to determine which self products of the H-2 complex if any are involved in recognition; (d) examine whether it is possible to generate cytotoxic effector cells by stimulation with TNP-modified soluble proteins, and if so, to determine whether such effectors are H-2 restricted; (e) map the immune response genes which control the ability to generate effector cells specific for the H-2D^{d} TNP restricted cytotoxic response; and (f) determine whether the clones of T-lymphocytes recognizing the alloantigens of a particular H-2 haplotype include the clone or clones of cells that recognize the same alloantigens modified by TNP.

Methods Employed: The methods employed for specific objectives (a) through (e) are indicated below.

(a) The specificity contributed by the TNP group to the cytotoxic effector cell was further investigated by comparing the lytic potential of the effector cells generated by sensitization with syngeneic trinitrobenzene sulfonate-(TNBS-) modified stimulators (which binds to cell surface proteins by covalent linkage) and with cells modified with TNP-stearoyl-dextran (TSD) (which binds to cell surface lipoprotein by noncovalent forces). Specificity was also tested by comparing TNBS- and TSD-modified syngeneic cells for lysis by and cold target cell inhibition of effector cells.

(b and c) Secondary in vitro responses to TNP-modified syngeneic cells were generated by restimulation of primary sensitized cultures at 5 to 14 day intervals. Optimal conditions were determined for the secondary cytotoxic and proliferative responses. The H-2 restriction of both the secondary cytotoxic and proliferative responses as well as the lytic phase of the secondary cytotoxic response were determined by using TNP-modified congenic and recombinant secondary stimulator and target cells.

(d) Mouse spleen cells were sensitized with TNP-modified soluble proteins such as bovine gamma globulin (TNP-BGG) and bovine serum albumin (TNP-BSA). The effector cells generated were tested for specificity on TNBS-modified target cells of different H-2 haplotypes.

(e) Mouse spleen cells from different inbred and congenic strains all expressing the H-2D^{d} haplotype and different haplotypes at the K and various I subregions were compared for their ability to generate cytotoxic effectors against H-2D^{d}-TNP

(f) Mouse spleen cells were sensitized in a primary culture against allogeneic spleen cells. During the primary sensitization the alloreactive clones of lymphocytes were destroyed by the addition of bromodeoxyuridine (BUdR) and light exposure. The cultures were subsequently restimulated with the allo-antigen, TNP-modified syngeneic cells, or TNP-modified allogeneic cells, and the cytotoxic potential measured after the secondary cultures.

Major Findings: The major findings for specific objectives (a) through (e) are summarized below.

(a) Effector cells generated by sensitization with TNBS-modified spleen cells did not lyse nor were they blocked by TSD-modified, H-2-matched target cells which contrasts with TNBS-modified H-2-matched targets. Furthermore, TSD-modified cells were not able to sensitize syngeneic spleen cells, in contrast to TNBS-modified cells.

(b) Secondary cytotoxic responses were generated in vitro to TNP-modified syngeneic cells. The responses obtained were restricted to K and D self H-2 products both with respect to restimulation and lysis of modified targets.

(c) Secondary in vitro proliferative responses were obtained against TNP-modified syngeneic cells. H-2 homology was required between the TNP-modified primary and secondary stimulating cells at the K, D or I regions of the H-2 complex.

(d) Effector cells were generated in culture against TNP-BGG and TNP-BSA. These effector cells were restricted to lyse only TNBS-modified target cells sharing K and/or D region products with the responding cells.

(e) Mapping studies indicated that at least two genes control the response potential to H-2D^{d}-TNP--one mapping between I-A and I-J, and the other mapping to the left of I-A, possibly in the K region.

(f) Elimination of alloreactive clones by BUdR and light also resulted in the loss of ability of the lymphocyte cultures to respond to the same allogeneic cells modified with TNP.

Significance to Biomedical Research and the Program of the Institute: The project is of fundamental immunological interest in that it describes a major histocompatibility linked immunological phenomenon involving self recognition. The recognition of self MHC-coded structures in association with foreign antigenic determinants raises the possibility that self recognition is important in autoimmunity and possibly for virally-infected autologous cells. The finding that products of the MHC are important for the antigenic complex recognized as well as for determining immune response potential provides a working hypothesis for bifunctional MHC control of disease susceptibility in those examples of HL-A-associated diseases in man.

Proposed Course of Project: Investigation of this model will continue in order to determine: (a) whether different self MHC products are recognized by

proliferating and cytotoxic T cells; (b) the possible role of macrophages in these cytotoxic and proliferating responses and the possible "modification" of macrophage MCH-coded products for recognition of the TNP-modified soluble antigens; (c) whether immune response genes can be identified for specificities other than H-2D^d-TNP, and to map such genes; (d) whether immune response genes influence the proliferative response to TNP-modified self, and if so, to map such genes and determine if they are distinct from those controlling cytotoxic responses; (e) the Ly antigens expressed on the populations of proliferating and cytotoxic cells recognizing TNP-modified self antigens; and (f) whether MHC-restricted cell-mediated responses can be generated against natural antigens such as tissue-specific or tumor-specific antigens.

Publications

Shearer, G. M., Rehn, T. G. and Schmitt-Verhulst, A.-M.: Role of the murine major histocompatibility complex in the induction of cell-mediated lympholysis to chemically modified autologous lymphocytes. Transplant. Rev. 29: 222-248, 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05043-03 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

AKR Leukemic Lines With T and B Cell Characteristics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: M. M. Zatz IPA Investigator I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several unusual in vivo murine lymphoma lines were established and studied for surface and functional characteristics. The lines were derived from AKR/J strain mice and are noteworthy in that they exhibit characteristics of both T and B cells. When passaged into young normal AKR/J hosts, the splenic cells appear first like T-cell lymphomas and then acquire aspects of both T and B cells. Characteristics studied were Thy 1 antigen, Ly antigen, Ia antigen, Fc receptor, complement receptor, mitogen responsiveness, and surface immunoglobulin. The Thy^+ tumor cells in lymph node remain Ig negative. It is possible, 1) that tumor cells differentiate to Thy^+ Ig^+ cells in spleen, but not in lymph node or 2) that two tumor subpopulations exist, one of which grows in spleen and lymph nodes (Ig^-), while the other, (Ig^+) grows more slowly and selectively in spleen. Attempts are being made to determine whether these tumor cells are differentiating, and if so, what the factors are which control this differentiation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this project is to investigate and characterize several unusual murine leukemias derived from spontaneous tumors arising in thymectomized and/or old AKR mice. This system was selected as a model for a rarely occurring natural variant of AKR leukemia in which the host animal has an atrophic thymus rather than the usual thymoma. Since these tumor cells express both T and B-cell markers, they provide a possible model for study of T and B cell differentiation.

Methods Employed: Passage lines of AKR tumors were established and continued by intravenous injection of 10^5 spleen cells into young AKR hosts. Donor cells were initially derived from tumors arising in old animals, some of which had been totally thymectomized at one month of age. Thus far, 4 lines have been well characterized: AKTB1, 2, 3, and 6. Several additional lines are now being studied.

Cells were studied for various characteristics including Thy antigen, Ly antigen (by absorption testing, surface immunofluorescence and cytotoxicity), surface immunoglobulin (SIg), in vitro synthesis of immunoglobulin, mitogen responsiveness and antibody-dependent cell-mediated cytotoxicity (ADCC). Fc receptor, complement receptor and Ia antigens were also studied. Kinetic studies on the acquisition of the above markers in vivo were carried out, with preliminary attempts to determine the factors regulating their expression.

Major Findings: 1) Thy and Ly antigen are present on 90-100% of spleen and lymph node (LN) cells of 3 AKTB tumor lines. Thy was best demonstrated using CBA anti-AKR anti-thy serum from hyperimmunized animals counterstained with fluorescein-labelled rabbit anti-mouse gamma$_2$ immunoglobulin. The anti-Thy sera used were carefully absorbed to remove anti-tumor and anti-B-cell reactivity. AKTB-1 is Ly 2.1^+; AKTB-2 and AKTB-3 are Ly 1.2^+.

2) SIg is demonstrable on 80 to 90% of AKTB spleen cells by the end of each passage. This SIg is of a single class - μ - as demonstrated by specific immunofluorescence and biosynthetic studies, and does not react with anti-alpha chain or anti-gamma chain labelled antibody. Similar results were obtained using polyvalent anti-immunoglobulin.

3) Within each passage of the AKTB-1 tumor line the percentage of SIg-positive spleen cells increases progressively from 15% (around 10 days) to 80-100% (around 24 days) at times when spleens are grossly enlarged (5-10 fold) and cells are Thy-positive. AKTB-6 tumor cells achieve a maximum of 30% Ig positive.

4) Lymph node and thymus cells from tumor-bearing animals with 90% SIg-positive spleen cells and gross lymphadenopathy are Thy-positive and SIg-negative.

5) Simultaneous staining for SIg and thy antigen reveal significant numbers (more than 50%) doubly marked cells in the spleen.

6) Capping and regeneration, in vitro culture, and biosynthesis studies all demonstrate that the Thy antigen and surface immunoglobulin are endogenously synthesized and not passively acquired. The immunoglobulin of the cell surface is monomeric IgM of the classical B-cell type.

7) Mitogen studies revealed that the DNA synthesis of AKTB1 cells (late in passage, SIg-positive) was increased in response to LPS but was suppressed by PHA or Con A. AKTB1 spleen cells early in passage (when cells were thy-positive and SIg-negative) and AKTB1 cells in lymph nodes responded to PHA and Con A but not to LPS.

8) Ia antigens and Fc receptors are present on AKTB spleen cells and their presence correlates with the presence of SIg on these cells. Lymph node cells are Ia and Fc receptor negative. The Ia antigens can be shown to be synthesized by the tumor cells. The Fc^+ tumor cells function in antibody-dependent cell cytolysis.

9) These tumors are indistinguishable from thymoma derived AKR lymphomas in their expression of ecotropic and xenotropic viral antigens.

Significance to Biomedical Research and the Program of the Institute: The results of these studies bear upon a number of issues of fundamental importance to both cellular immunology and also to viral leukemogenesis:

1) There are very few cell lines which bear easily detectable T and B cell markers, although a normal subpopulation with such characteristics has been identified in small numbers. This line may represent an abnormal proliferation of this population or an arrested phase of normal lymphoid cell development, implying transitions between T and B cells.

2) The SIg on these cells may represent an abnormal amount of the "normal" T cell immunoglobulin, so that this line could provide a way to examine "IgT" and the putative T cell antigen receptor. Preliminary studies indicate, however, that the membrane Ig is of the classical "B" cell type.

3) The difference between spleen and lymph node tumor cells in the expression of SIg within the same animal suggests that this system may be helpful in studying the effect of "microenvironment" on differentiation of tumor cells within the lymphoid system.

4) The ability to isolate SIg from these cells may allow the generation of anti-idiotype antibody in another species, and thus answer the question of whether or not AKR leukemia is a monoclonal disease.

5) Since the AKTB lines bear B cell markers, they may provide a system in which to examine B cell differentiation.

6) Since these doubly marked tumors occur selectively in thymectomized mice or old mice with atrophic thymuses, they may provide information on the target cells for leukemogenesis in a system where the thymic influence is reduced.

Proposed Course of Project: 1) Thus far, four AKR lymphomas have been well characterized with both T and B cell markers. Several additional peripheral lymphomas have arisen in old AKR mice without the usual thymoma. These tumor lines will be studied for their T and B cell markers in order to determine whether doubly marked lymphomas represent a more frequent biological phenomenon. If these dually marked tumors do represent a stage of normal cell differentiation it will be important to have multiple lines available to study differentiation pathways.

2) The most intriguing question which these tumors pose, is whether or not they may serve as a model for T-, B-, and/or stem cell differentiation. At least two of the TB tumor lines initially appear in spleen as Ig^-, Thy^+, but become Ig^+, Thy^+, while tumor cells in lymph node and thymus remain Ig^-, Thy^+. These findings may be explained by 1) a stable mixture of two tumors which grow at different rates selectively in spleen and lymph node and/or 2) the differentiation of Thy^+ Ig^- cells in spleen to Thy^+, Ig^+ cells, and the block of this step by the lymph node microenvironment. In order to explore these questions further, splenic tumor cells will be fractionated into Ig^+ and Ig^- populations by cell separation techniques, including use of the FACS. These populations will be transferred in vivo and placed in vitro in order to assess the factors which control acquisition of B-cell markers by these subpopulations. Attempts will be made to adapt these tumor lines to growth in vitro so that their expression of T and B cell markers can be studied as a function of the phase of the cell cycle.

Publications

Greenberg, R. S., Mathieson, B. J., Campbell, P. S. and Zatz, M. M.: Multiple occurrence of spontaneous AKR/J lymphomas with T and B-cell characteristics. J. Immunol. 118: 1181-1190, 1977.

Hurwitz, E., Zatz, M. M. and Segal, D.: The biological activity of affinity cross-linked oligomers of rabbit immunoglobulin G as tested by antibody dependent cell-mediated cytolysis (ADCC). J. Immunol. 118: 1348-1353, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05045-03 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Nitrophenyl-Modified Syngeneic Lymphocyte Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. M. Shearer Senior Investigator I NCI

COOPERATING UNITS (if any)

J. K. Inman, Laboratory of Immunology, NIAID

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The specificity of C57BL/10 cytotoxic effector cells generated by in vitro sensitization with autologous lymphocytes modified with a series of related nitrophenyl compounds was investigated. Effector cells generated by sensitization with autologous cells modified by the trinitrophenyl (TNP) group did not lyse target cells in which the TNP group was separated from the cell surface by a tripeptide spacer group. The same effectors lysed targets in which the TNP group was coupled directly to the cell surface. These results demonstrate the high degree of fine specificity exhibited by these effector cells, and indicates that there is not a distinct receptor on the cytotoxic T-lymphocyte which recognizes the TNP moiety exclusively.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this series of experiments was to determine how specific T-cells for cytotoxic function were in their ability to discriminate between slight alterations in the TNP group when attached to syngeneic cells.

Methods Employed: Nitrophenyl compounds were prepared having a reactive group which would link to cell surface amino groups. These compounds were used to modify mouse spleen cells and the modified cells were used to sensitize syngeneic lymphocytes leading to the generation of cytotoxic effector cells. The effector cells were tested for their specific cytotoxic reactivity using the ^{51}Cr-release assay on H-2 matched targets modified with these compounds.

Major Findings: The cytotoxic effector cells generated were able to discriminate between target cells in which the TNP moiety was directly attached to cell surface amino groups from those in which TNP and the cell surface were separated by an alanylglycylglycyl tripeptide spacer.

Significance to Biomedical Research and the Program of the Institute: This project is of basic immunological interest in that it demonstrates the high degree of specificity exhibited by T-lymphocytes, and could be useful for models of autoimmunity and the possible role of the major histocompatibility complex in such reactions.

Proposed Course of Project: This project is being terminated as a separate project. Any further research dealing with the fine specificity of "hapten" modified self reactions will be included in Project No. Z01-CB-05038-05-I.

Publications

Rehn, T. G., Inman, J. K., and Shearer, G. M.: Cell-mediated lympholysis to H-2 matched target cells modified with a series of nitrophenyl compounds. J. Exp. Med. 144: 1134-1140, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05048-03 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Attempted Isolation of Fc Receptor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler Senior Investigator I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolation of the Fc receptor from detergent solubilized radiolabelled murine lymphoid cells was attempted using Ig complexes of various types. No component(s) of the lymphocyte bound specifically to the complexes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Immunochemical characterization of Fc receptors.

Methods Employed: Spleen cells were radiolabelled with ^{3}H-leucine, and the labelled cells were detergent solubilized. The detergent-soluble material was then used as a putative source of labelled Fc receptor.

Binding to immobilized IgG aggregates was found unsatisfactory due to nonspecific absorption problems. Binding to immune complexes formed between TNP-KLH and affinity purified anti TNP antibodies was tested.

Major Findings: The binding to immobilized IgG aggregates was pursued for some time and attempts were made to reduce the non-specific absorption effects. This technique was eventually abandoned however because it could not be rendered specific.

Results using immune precipitates showed binding of material in the molecular weight range of 60-70,000 daltons and 15-20,000 daltons. However, these components were not specific for Ig complexes.

Significance to Biomedical Research and the Program of the Institute: Fc receptors are a potentially important molecule on the lymphocyte surface with regard to immune function. As such it is of great interest to isolate it, characterize it and raise specific antibodies directed towards it.

Proposed Course of Project: Recent evidence from other laboratories indicates that Fc receptors may have very low binding affinities. In view of this information we intend to approach the isolation of Fc receptors via the use of affinity labels to bind the Ig complexes covalently to Fc receptors prior to solubilization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05049-03 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

F_1 Antiparental Cell-Mediated Lympholysis In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	C. M. Pettinelli	Visiting Fellow	I NCI
	A.-M. Schmitt-Verhulst	Expert	I NCI

COOPERATING UNITS (if any)

G. Cudkowicz, Department of Pathology, State University of NY, Buffalo, NY;
H. Wigzell, Department of Immunology, Uppsala University, Uppsala, Sweden;
R. Kiessling, Department of Tumor Biology, Karolinska Inst., Stockholm, Sweden

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.4	0.6

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse F_1 spleen cells can develop cytotoxic effector cells in vitro directed against parental cells. The antigenic differences detected appear to be controlled by a structural gene (Hh-1) mapping in or near the D region of the H-2 complex. Although a number of positive correlations have been demonstrated between murine hemopoietic graft rejection in vivo and F_1 anti-parent cytotoxicity in vitro, certain differences have been recently found between these two F_1 anti-parent reactions. These include the observations that marrow graft rejection does not require priming and is not dependent upon thymus-dependent cells, whereas the development of cytotoxic cells requires sensitization and is effected by T-lymphocytes. Furthermore, the cytotoxic effector cells appear to require the recognition of more than Hh-1, possibly other self antigens associated with cytotoxic function. Comparisons were also made between natural killer (NK) cell activity and F_1 anti-parent marrow rejection in vivo and cytotoxic activity in vitro. A number of parallels were observed among NK cell activity, F_1 anti-parent marrow graft rejection in vivo, and F_1 anti-parent cytotoxicity in vitro.

Project Description

Objectives: The objective of this project was to investigate the immunogenetic parameters associated with the generation of murine F_1 anti-parent cytotoxic effector cell in vitro, and to compare them with allogeneic cytotoxicity in vitro, NK cell activity in vitro, and F_1 anti-parent hemopoietic graft rejection in vivo.

Methods Employed: Spleen cells from a number of F_1 mouse strains were sensitized in culture against parental spleen cells. The effector cells generated were assayed on ^{51}Cr-labelled target cells expressing different H-2 haplotypes. NK cell activity was tested in the spleens of mice and a number of parameters were compared with F_1 anti-parent reactivity.

Major Findings: Although a number of similarities have been observed between F_1 anti-parent marrow graft rejection in vivo and the development of F_1 anti-parent cytotoxic effector cells in vitro (see previous annual report), a number of differences were also noted. These include the findings that: (a) the in vivo rejection process does not require active immunization, whereas the development of cytotoxic effectors does; (b) marrow graft rejection is neither effected by nor dependent upon thymus-derived cells, whereas the F_1 anti-parent effectors and their precursors are T-cells; and (c) the recognition responsible for F_1 anti-parent graft rejection appears to involve structures coded for by a gene(s) mapping near H-2D, whereas for F_1 anti-parent cytotoxicity, products of additional genes within the H-2 complex are also important. The comparison of NK cell activity in vitro with F_1 anti-parent marrow graft rejection in vivo and F_1 anti-parent cytotoxicity in vitro indicated that all three phenomena probably share a common cell type which may serve an accessory role in each type of response.

Significance to Biomedical Research and the Program of the Institute: These comparative studies may be of importance for understanding the common and distinct immune pathways for these three phenomena. Such an understanding may be of significance since marrow graft rejection is considered a problem of transplantation, NK cell activity is considered to be an important mechanism for surveillance against lymphoid tumor development, and the cytotoxic response is mediated by a classical T-lymphocyte mechanism.

Proposed Course of Project: The comparative study of these three models will be continued, and various cell types will be studied for their possible contribution to the response. Further mapping studies using recombinant mouse strains will be made in an attempt to map the Hh-1 gene within H-2, as well as to find the additional H-2 region(s) important for F_1 anti-parent cytotoxicity.

Publications

Shearer, G. M., Cudkowicz, G., and Garbarino, C. G.: In vitro induction of F_1 hybrid anti-parent cell-mediated cytotoxicity. J. Immunol. 117: 754-759, 1976.

Shearer, G. M., Waksal, H., Cudkowicz, G.: An in vitro model of hybird resistance to bone marrow grafts. Transplant. Proc. 8: 469-475, 1976.

Shearer, G. M., Cudkowicz, G., Schmitt-Verhulst, A.-M., Rehn, T. G., and Evans, P. D.: F_1 hybrid anti-parental cell-mediated lympholysis: A comparison with bone marrow graft rejection to Hh determinants in vivo and with cell-mediated lympholysis to H-2 alloantigens in vitro. Cold Spring Habor Symp. Quant. Biol. (Proceedings of meeting on Origins of Lymphocyte Diversity, June 1-8, 1976.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05050-03 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Structure and Function of the Constant Portions of Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. M. Segal	Chemist	I NCI
OTHER:	E. Hurwitz	Visiting Fellow	I NCI
	E. Alexander	Guest Worker	I NCI
	M. Zatz	Visiting Fellow	I NCI
	H. Metzger	Chief, Chem. Immunol.	NIAMDD
	J. Taurog	Research Associate	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study was undertaken to investigate the interaction of immunoglobulins with various cell surface receptors and immune amplification molecules. Covalent complexes have been prepared by specifically cross-linking antibody molecules through antigen interactions at their combining sites and nonspecifically by chemically cross-linking lysines of neighboring antibody molecules. Oligomers of defined size have been isolated. We have been able to define the affinities and kinetics of binding of IgG and IgG oligomers to the Fc receptors of various cell types. In addition, subclass specificity for murine IgG myeloma proteins has been determined. Preliminary studies of the binding properties of isolated populations of human cells have been carried out. The binding properties of oligomers have been correlated with their ability to block antibody dependent cell mediated cytolysis using three populations of effector cells.

Cross-linked proteins of the IgE class have been used to show that the allergic triggering unit for mast cells consists of two cross-linked IgE molecules. The fixation of complement by monomeric IgG has been characterized and studies on the interaction of the complement system with IgG oligomers have been initiated.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To investigate the interactions of the constant parts of immunoglobulins with the various effector systems employed in immune reactions; 2) To study the relationship of antigenic recognition to these interactions and to study the molecular basis of these phenomena.

Methods Employed: Organic synthesis, gel filtration, ion exchange chromatography, polyacrylamide gel electrophoresis, complement fixation, radiolabelling of proteins, tissue culture, binding assays, antibody-dependent cytotoxicity assays, cell separations.

Major Findings: 1) The binding of affinity cross-linked oligomers of rabbit IgG antibodies to macrophage- and lymphocyte-like murine cell lines and to normal mouse spleen cells has been investigated in detail. Binding constants for oligomers of different sizes have been measured, and it has been concluded that receptors for IgG on all cell types examined in this study interact weakly with rabbit IgG. Oligomers of IgG bind more tightly than the monomeric protein but the free energy of interaction is not an additive function of the number of Fc subunits present in each oligomer. The data suggest that the simultaneous binding of all Fc units of an oligomer to cell-bound receptors leads to interactions which have a destabilizing effect upon the bound complex. An alternative explanation would suggest that the receptors on the cell surface bind to the oligomer independently, as for example hydrogen ions would bind to a dicarboxylic acid. This latter interpretation would result if the cell bound receptors had nearly the freedom of movement as if they existed free in solution.

The kinetics of interaction between trimeric IgG and cells have also been measured. Both "on" and "off" reactions occur in multiple steps. The rate of loss of bound oligomers is particularly sensitive to oligomer size; the larger the oligomer the slower the loss of bound oligomer.

Monomeric IgG at concentrations present in normal serum inhibited the binding to cells of oligomers and antigen-antibody complexes. Monomeric IgG also increased the exchange rate of bound oligomer, and a model for the interaction of oligomers with cells has been proposed.

The results suggest that natural antigen-antibody complexes will bind weakly to cells in vivo because of the large amount of ambient IgG. It seems possible that IgG receptors are normally used in conjunction with other receptors, and that only the largest complexes would bind to Fc receptors by themselves.

2) Binding of IgG by Fc receptors is both cell and protein specific. Certain cells bind Ig while others do not.

We have investigated the class and subclass specificity for immunoglobulin binding in the mouse using macrophage-like and lymphocyte-like tumor lines, and normal spleen cells. The results show conclusively that the three major subclasses of murine IgG (IgG1, IgG2a, IgG2b) all bind to the same receptors

in all three cell types, and that the affinities are identical within experimental error. IgG3 proteins bind much more weakly, and proteins of the IgM and IgA classes do not bind to the IgG site. The pK_d for monomer binding (at 0°) is 5.7 $\pm$ .4 for IgG1, 2a, and 2b for the three cell types.

3) We have also begun looking at the binding of rabbit IgG to human cells. Fc receptors have been detected in human mononuclear leukocyte populations, lymphocyte populations (depleted of macrophages) and on polymorphonuclear cells. In all cases the association constants for the binding of rabbit IgG are similar to those found in mice. The number of receptors per cell average 10^4 - 10^5 for lymphocyte and mononuclear leukocyte populations and is about 10^6 for the polymorphonuclear leukocytes.

Immunoglobulin receptors have not been detected on tonsillar lymphocytes nor thymocytes. Cells bearing Fc receptors have been detected in human peripheral blood and splenic mononuclear leukocytes.

Preliminary studies in which cells bearing surface IgM are removed by immunoadsorption indicate that in the peripheral blood, those cells which have Fc receptors lack surface IgM.

4) The function of the Fc receptor has been probed by using rabbit IgG oligomers to block antibody-dependent cell-mediated cytolysis (ADCC). The system used measures the lysis of chicken red cells coated with rabbit anti-chicken erythrocyte antibody. Lysis was carried out by cells from a lymphocyte line, a macrophage line and by normal spleen cells.

It was observed, first of all, that cytolysis was mediated only by those cells which had Fc receptors on their surfaces. The lymphocyte line has two sublines -- a spleen derived line and lymph node line. When tumor cells from the same animal were examined, the spleen cells had Fc receptors on their surfaces, while the lymph node cells did not. As expected, only the splenic derived cells would serve as effector cells in an ADCC reaction.

Blocking of ADCC when the macrophage tumor cells were used as effectors correlated well with oligomer binding. That is, when approximately 50% of the receptor sites were coated with dimeric IgG, ADCC activity was blocked to roughly the same extent. Results with this cell line could thus be interpreted that blocking of receptor sites on the cells with soluble IgG oligomers prevented the effector cells from interacting with the antibody-coated target cells. The results also show that the interaction of macrophage-like tumor cells with the target cells is not measurably stronger than the interactions between effector cells and small oligomers. If it were, the oligomers would not have been able to block cytolysis.

When cells of the lymphocyte line were used as effectors, blocking of ADCC occurred under conditions in which a small fraction of receptor sites should be occupied. Since binding measurements were not carried out simultaneously with the ADCC reaction (due to technical problems) we were not sure in this case that binding and inhibition did not parallel each other. It is also

possible however, that the oligomers could generate a signal which prevents ADCC from occurring with this cell line.

5) Preliminary studies on the interaction of oligomers with the complement system have been carried out. We have shown that under certain circumstances, monomeric IgG can cause complement fixation. This appears to be caused by a serum protein which attaches to IgG during preparation, and directly activates C2.

When oligomers are prepared from a non-binding monomeric preparation none of the oligomers are observed to fix complement in a standard fixation assay. We are currently investigating whether this is also true when other assays are employed with the purpose of determining whether monomeric, dimeric, trimeric, and tetrameric IgG are really inert with respect to the complement system under physiological conditions.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by immunoglobulins elicits a number of reactions which normally lead to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An ultimate goal is to enhance the immune response toward neoplastic cells.

Proposed Course of Project: Binding of immunoglobulins to human cells will be characterized in detail. We have recently discovered that proteins of the IgM class also bind to a receptor distinct from that binding IgG on a wide variety of cells and we have begun to characterize this phenomenon. Functional studies for the purpose of defining the role of Fc receptors in controlling antibody production have been planned.

We have also begun a collaboration with Dr. Brian Tack (NIAMDD) to characterize the binding of C3 to several cell types.

We are also hoping to further characterize the effects of oligomerization on the complement system, and perhaps with immune clearance.

Publications

Segal, D.M. and Hurwitz, E.: Dimers and trimers of immunoglobulin G covalently cross-linked with a bivalent affinity label. Biochemistry 15: 5253-5258, 1976.

Segal, D.M. and Hurwitz, E.: Binding of affinity cross-linked oligomers of IgG to cells bearing Fc receptors. J. Immunol. 118:1338, 1977.

Hurwitz, E., Zatz, M.M., and Segal, D.M.: The biological activity of affinity cross-linked oligomers of rabbit immunoglobulin G as tested by antibody dependent cell-mediated cytolysis (ADCC). J. Immunol. 118: 1348, 1977.

Segal, D.M., Taurog, J.D., and Metzger, H.: Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. Proc. Natl. Acad. Sci. USA, 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05051-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Spontaneously Arising Syngeneic Murine Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	R. B. Levy	Postdoctoral Fellow	I NCI
	T. H. Hansen	Postdoctoral Fellow	I NCI
	S. D. Waksal	IPA Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An AKR/J murine carcinoma-like tumor was generated in tissue culture from an undifferentiated fibrosarcoma. An autologous immune response was generated against the carcinoma in vivo and in vitro. Subcutaneous injection of low numbers of the carcinoma cells resulted in a period of tumor growth followed by tumor regression. Lymphocytes from regressing mice were specifically cytotoxic for carcinoma target cells. Cytotoxic effector cells could also be generated in vitro against the carcinoma, and the injection of cells containing effectors protected host mice from a lethal inoculum of the tumor. T-cell-mediated and antibody-mediated cytotoxic studies were performed to determine if new antigens were expressed on the carcinoma cell line. The results from both types of experiments suggested that a loss of a private H-2D^{k} specificity had occurred with a concomitant gain of a private H-2D^{d} specificity. Rejection of the tumor in vivo and the generation of cytotoxic effector cells in vitro was shown to be associated with this loss and gain of major histocompatibility complex (MHC) controlled antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project were to investigate the immunogenetic parameters associated with the rejection by mice from the strain of origin of a cultured tumor cell line. The specific objectives were to:

(a) Establish if the observed regression of the tumor cell line by syngeneic mice had an immunological basis, and to determine if cytotoxic effector cells could be generated in vivo and/or in vitro against the tumor;

(b) Test whether cells from sensitized cultures would specifically protect syngeneic mice against a lethal inoculum of the tumor;

(c) Determine whether the immune response generated against the tumor could be attributed to antigenic changes in the tumor cell line, and if so, to test whether such antigenic changes were associated with products of the MHC and to map these changes within the H-2 complex.

Methods Employed: The methods employed for (a) through (c) were:

(a) Lymphocytes from tumor regressing mice were tested for specific cytotoxic activity and for their potential to generate enhanced cytotoxic responses following secondary stimulation in vitro using a 5-hour ^{51}Cr-release assay on a variety of target cells.

(b) Normal AKR/J mice were injected with lymphocytes containing cytotoxic effector cells as well as with numbers of carcinoma cells which result in death of the mice, and the number of mice surviving was determined.

(c) Spleen cells from normal AKR/J mice were sensitized against a number of stimulating cells differing from the AKR/J responding cells at specific regions or subregions of the H-2 complex. The effector cells generated were tested on a series of target cells differing at specific regions of H-2 using the ^{51}Cr-release assay. Antibody-mediated, complement-dependent lysis of the carcinoma tumor cell line was performed using H-2 region specific antisera.

Major Findings: The major findings are:

(a) During the regression of the carcinoma tumor, syngeneic AKR/J mice were found to have lymphocytes which specifically lysed the carcinoma tumor. Cytotoxic effector cells which lysed the carcinoma could be generated in vivo or in vitro.

(b) AKR/J lymphocytes sensitized in vitro against the carcinoma were capable of protecting AKR/J mice against lethal inoculation of the tumor.

(c) Antigenic changes were detected in the cultured carcinoma cell line by both cellular and antibody-mediated immune reactions. A loss of the normal AKR/J H-2D^{k} private specificity was detected with a concominant gain of an H-2D^{d} specificity.

Significance to Biomedical Research and the Program of the Institute: This project is of basic importance for tumor immunology as well as for the genetic expression of MHC controlled cell surface antigens. These findings raise the possibilities that: (a) tumor viruses may be associated with the regulation and/or deregulation of cell surface antigen expression; and (b), an animal strain expressing a particular MHC haplotype may have the genetic information for a number of haplotypes but the expression of a particular haplotype may be influenced by regulator genes mapping within the MHC.

Proposed Course of Project: The carcinoma cell line will be tested for other AKR/J cellular markers which are not linked to the H-2 complex such as enzymes in order to test by independent methods whether the cells are of AKR/J origin or possibly of A-strain origin, which could have occurred by contamination. The antigens on the cell surface will be analyzed biochemically to determine how similar they are to known H-2D^{d} antigens. Attempts will be made to isolate virus from the cell line and to use the virus to infect and transform other AKR/J cell lines. Other AKR/J tumor cells as well as noeplastic cells from other strains will be studied to determine if the antigenic "switches" seen in the carcinoma line are more general.

Publications

Levy, R., Waksal, S., and Shearer, G.: Correlation of suppressor cell development in parental and F_1 hybrid mouse strains with the growth of a parental tumor in vivo. J. Exp. Med. 144: 1363-1368, 1976.

Levy, R., Waksal, S. and Shearer, G.: Control of immunity in parental and F_1 hybrid mouse strains to a spontaneously arising parental tumor. I. Correlation between in vivo and in vitro anti-tumor reactivity in responsive and unresponsive animals. Eur. J. Immunol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05052-02 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Psychosocial Factors in the Prognosis of Melanoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. N. Rogentine	Senior Investigator	I NCI
OTHER:	J. Rosenblatt	Clinical Associate	AP NCI
	D. P. Van Kammen	Chief, Unit on Neuropsychopharmacology	AP NCI

COOPERATING UNITS (if any)
W. Bunney, NIMH; B. Fox, W. Terry, S. Rosenberg, and R. Fisher, NCI

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the effect of various psychosocial factors on host resistance to malignant melanoma. A detailed characterization of these factors in patients with melanoma, Clinical Stage II, is being obtained. In addition certain endocrine and immune parameters are also being studied. We are testing several hypotheses which may distinguish those patients suffering recurrence of their disease from other who either remain disease-free or have a delayed recurrence. At one year, significant correlations with disease free status were found with 1) number of positive lymph nodes, 2) ABO type, 3) social assets, and 4) melanoma adjustment self rating score (see text).

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Malignant melanoma is an unpredictable disease. Its course can range from rapid demise to spontaneous remission. The reasons for this variability are not known.

Psychosocial factors are known to play a role in the development of a wide variety of medical illness: e.g., duodenal ulcer and coronary heart disease and there is a growing body of evidence that such factors predispose normal individuals to malignancy, or modify its course once established. It is thus conceivable that psychosocial factors may account for some of the variability in the clinical course of malignant melanoma.

Clinical Stage II melanoma is defined as a local primary malignancy with extension to regional nodes. Surgical resection can render these patients disease-free. Many of such "cured" patients subsequently develop Clinical Stage III disease (distant metastases), usually within two years and go on to die. For a period of time, however, there exists a unique group of patients with known cancer, potentially cured, but at risk of recurrence. Since much previous psychosomatic work has been retrospective in type and fraught with the inherent methodological flaws of that approach, this patient group presents a uniquely important opportunity to carry out a well controlled prospective study on the influence of psychosocial factors on disease progression.

Our purpose is to develop a detailed characterization of and to test several hypotheses regarding the psychosocial factors which distinguish those patients who suffer recurrence of the malignancy from those who either remain disease-free or who have a delayed recurrence.

Methods Employed: We plan to employ the following methods in generating a psychosocial profile of these patients.

I. Psychiatric Interview -

Each patient will be seen following surgical recuperation or during clinical staging for an interview of approximately an hour and a half duration. The purpose of this interview will be to gather data regarding the patient's past childhood, occupational and marital history, to identify the relevant psychosocial factors possibly involved in precipitating the current illness, and to make an assessment of the patient's attitude toward the future, his involvement, integrity of ego functions and object relations.

II. Standardized Questionnaire -

1. MMPI - This is an empirically derived and extensively studied instrument which generates a profile of the patient in several dimensions of personality. This test has very high reliability and will be administered once.

2. Holmes and Rahe Schedule of Recent Experience - This is a 52-question form used to assess the magnitude of life change the patient has experienced during a given time interval. It has had the benefit of extensive validation study and previous research has demonstrated a high correlation between the magnitude of life change measured by this instrument and the onset of subsequent illness. This scale will be administered initially and at 2 month intervals.

3. SCL-90 Symptom Checklist - This is a self-administered form which checks for the presence of a wide variety of psychological and neurovegetative symptoms. It will be administered initially and at 2 month intervals.

4. Rotter Internal - External Locus of Control Scale - This is a 29-item self-administered form which is currently being widely researched. It appears to be a personality dimension with correlates from the neurological to social level of organization. This test also has very high reliability and will be administered only once.

III. Psychobiological Measures -

The concurrent intensive study of the immune system being conducted with these patients will also allow us to gather data which may suggest a mediating mechanism for the effect, if any, of psychosocial factors on the progression of the disease. It is possible that psychosocial stress may modify immune responses, for example, via at least two stress responsive hormones, cortisol and the catecholamines. Both these hormones modify immune responses. Since the immune system seems to play a role in the development and containment of cancer, psychosocial factors may influence malignancy via the intermediary of the immune system.

In addition to the assays of immune competence we will collect on the initial admission 24-hr urine samples for 17 hydroxycorticosteroid and 17 ketosteroid levels, and VMA excretion.

Once these tests are conducted and scored, the results will be correlated with the patient's stage of disease progression. Our aim is to construct a psychosocial and psychobiological profile of "progression" and "non-progressors".

Major Findings: To date we have entered 63 patients into the study. This report concerns the first 31 patients studied. Of this group 18 relapsed within one year after surgery (median time to relapse=223d); 13 remained disease free.

Four variables correlated significantly with the one-year disease-free status. In response to the first question of the Holmes and Rahe Recent Life Changes Questionnaire, relapsers weighted on a scale of 1 to 100 the amount of personal adjustment necessitated by their having melanoma at 52 ± 30 (med.=50). Non-relapsers rated their melanoma adjustment at 83 ± 17 (med.=90), $p<.005$. Ratings on the Luborsky Social Assets Scale were higher in relapsers, $p<.05$. Patients

with blood group O tended to relapse (16 of 20), whereas those with blood group A tended to stay in remission (only 2 of 10 relapsed), $p<.01$. Finally all 9 patients with seven or more nodes positive for metastases relapsed, whereas only 9 of 22 with less than 7 nodes positive relapsed, $p<.05$.

These preliminary findings will form a basis for a prospective study of the outcome of a subsequent group of patients. They seem to suggest that some psychosocial and genetic factors as well as biological are important in the short term prognosis of melanoma.

Significance to Biomedical Research and the Program of the Institute: An understanding of all environmental and host factors in oncogenesis is necessary to progress in cancer research. Psychosocial factors can influence disease occurrence and prognosis. The magnitude of the impact of these factors on host resistance to neoplasms has never been adequately documented, in large part due to the retrospective design of previous clinical experiments. This study avoids that problem: it is a prospective study. Thus we shall be able to make stronger statements about possible psychosocial correlates with cancer upon its completion. Identification of psychological or social stress factors in the promotion of tumor growth is clearly of importance basically as well as for its therapeutic implication. Just as immunotherapy is designed to strengthen host resistance to tumor, psychotherapy could conceivably be used for the same reason.

Proposed Course of Project: We propose to enter about 100 patients into the study. The data from the first 31 patients has been analyzed and will be the basis of hypotheses which the next group of patients will test.

If clear cut correlations between certain psychosocial variables and disease progressions are found, we will consider studying appropriate psychotherapeutic measures in the treatment of melanoma.

In addition any correlations between psychosocial factors and endocrine or immune variables will be sought and appropriate further clinical experiments designed to investigate their nature more fully.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05053-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Resistance to AKR Leukemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. M. Zatz	IPA Investigator	I NCI
OTHER:	A.-M. Schmitt-Verhulst	Expert	I NCI
	G. M. Shearer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(AKR/JxDBA/2J)F_1 and H-2 compatible AKR/Cu mice resist transplantation of spontaneous AKR/J lymphoma. Resistance correlates with the development of cell mediated lympholysis (CML) in vitro. A primary F_1 anti-AKR CML response results in Thy^+ effector cells which lyse normal or lymphoma cells, suggesting that parental rather than tumor antigens are involved. The results suggest that "Hh" antigens are expressed on, and play a role in rejection of, spontaneously arising lymphoma cells. In vitro CML response between AKR substrains occurs only after in vivo priming with normal or lymphoma cells. The secondary in vitro CML can be elicited with, and assayed on, normal or tumor cells. Although the only known antigenic difference between the AKR substrains is the Thy antigen, B and T cells are equally good as immunogens and targets. Thus, the CML response is not directed solely to the Thy antigen. This CML is H-2 restricted for responder, immunogen and target cells. The data suggest the existence of a minor H antigen linked to the Thy locus. The AKR substrains differ genetically only at a region of chromosome 9, express high titers of murine leukemia virus and have 80-90% incidence of spontaneous lymphoma, yet a limited and possibly definable antigenic modification results in rejection of the reciprocal strains lymphoma.

Project Description

Objectives: The objectives of this project are to define the basis for resistance or susceptibility to growth of AKR lymphoma cells in the high leukemia incidence strain of AKR/J mice, F_1 hybrids of AKR/J, and H-2K compatible mice.

Methods Employed: 1. In vivo resistance. The growth of AKR/J lymphoma cells in AKR/J, AKD_2F_1/J, AKR/Cu and CBA/J mice were studied. Doses of 10^3-10^6 lymphoma cells from spleen or thymus of AKR/J mice with spontaneous lymphocytic leukemia were injected i.v. into the recipients. Survival was compared between groups. Resistance of AKR/J mice to transplantation of spontaneously arising AKR/Cu lymphoma cells was also investigated.

2. In vitro resistance. Spleens of normal young mice, or of mice previously challenged with AKR/J lymphoma cells, were assayed in vitro for cell-mediated lympholysis (CML). Cells were cultured in vitro for 5 days with appropriate ratios of 2000R irradiated cell immunogens. After 5 days the cells were harvested and assayed for their ability to kill ^{51}Cr labelled tumor targets (RDM4, derived from AKR/J) or appropriate PHA-stimulated spleen lymphocytes.

Major Findings: 1. AKD_2F_1 resistance: $AKRxDBA_2$ (H-2k, Thy 1.1 x H-2d, Thy 1.2)F_1 hybrids show selective resistance to growth of AKR/J (H-2k/Thy 1.1) lymphoma cells at doses of 10^3-10^5 cells. Survival is prolonged anywhere from 1 week to 1 month, depending on the experiment and dose employed; eventually AKD_2F_1 mice develop lymphoma and die.

2. AKR/Cu resistance. AKR/Cu mice, derived from the same common stock as AKR/J, have a mutation of chromosome 9, rendering them Thy 1.2 instead of Thy 1.1. Other changes are associated with this mutation, i.e., a difference in an esterase enzyme. These AKR/Cu mice, which resemble the AKR/J mice in their high titer of murine leukemia virus and spontaneous incidence of lymphomas, are resistant to growth of AKR/J tumor cells. At doses of 10^5 cells, 100% of mice survive the injection indefinitely; at doses of 10^6 cells, 80% of mice survive. The reciprocal experiment gives similar results, i.e., AKR/J mice survive indefinitely the injection of 10^5 AKR/Cu lymphoma cells.

3. CBA resistance. CBA mice which are H-2k, but Thy 1.2, also differ from AKR/J mice at multiple minor histocompatibility loci. These mice are even more resistant than are AKR/Cu mice to growth of AKR/J lymphoma. Survival of mice with injection of $5x10^6$ AKR/J lymphoma cells is 100%.

4. AKD_2F_1 anti-AKR/J CML response. AKD_2F_1 cells can be sensitized in vitro by normal and lymphoma cells. The responder cells can be inactivated by prior in vivo silica treatment; effector cells can be inactivated in vitro by anti-Thy serum treatment. The ontogeny of the F_1 anti-AKR/J response is later than the development of responsiveness to alloantigens. Thus the in vitro CML response resembles other known F_1 antiparent "Hh" responses and may reflect the in vivo basis of F_1 resistance to growth of parental lymphoma cells.

5. CML responses between AKR substrains. Secondary CML responses between AKR substrains occur following priming in vivo with normal or lymphoma cells. Normal lymphoma cells serve equally well as secondary immunogens in vitro and as target cells. Although the only known antigenic difference between AKR/J (Thy 1.1) and AKR/Cu (Thy 1.2) substrains is the Thy antigen, the present studies demonstrate that the in vitro CML is not related solely to Thy antigen expression. Thus T cells and B cells, as well as spleen and thymus cells, are equally effective as immunogens and targets. Primed AKR/J responder cells will be stimulated by and lyse C3H and B10.BR (H-2k, Thy 1.2) cells, but not CBA cells (H-2k, Thy 1.2), while primed AKR/Cu responder cells will be stimulated by and lyse CBA and B10.BR cells, but not C3H cells. These data, as well as skin graft rejection data, suggest that the in vitro responses between AKR/Cu and AKR/J mice are due to the presence of a minor histocompatibility antigen, previously unknown, which maps in the 9th chromosome.

Further studies show that the expression of the CML response is restricted by the H-2 type of immunogen and target cells. Thus primed AKR/Cu cells require immunogen and target cell homology at the D end of the MHC, while primed AKR/J responder cells require homology at either the D or K end of the MHC.

Significance to Biomedical Research and the Program of the Institute: The AKR/J mouse provides a model for study of acute lymphocytic leukemias of T-cell origin; possibly similar T-cell leukemias, presenting with thymoma, have been described in man. The question of leukemogenesis in AKR/J mice is complex. Several factors play critical roles in determining the onset and incidence of murine leukemia. These factors are 1) the levels of murine leukemia virus, 2) the H-2 type, and 3) the thymus epithelium. It is hoped that by comparing the resistance of the AKR/J mouse to related strains an understanding of the genetic basis of resistance to lymphoma may be achieved. These studies also offer the opportunity to pinpoint antigenic differences, expressed on lymphoma cells, which can elicit effective host immunity.

Proposed Course of Project: 1. F_1 resistance. The major emphasis will be on measuring changes in the level of CML following in vivo challenge with lymphoma cells. Since the mice initially resist growth of cells but eventually succumb, something may be learned of changes in level of immunity paralleling these in vivo events. Attempts will be made to modify host immunity by immunization and tolerization.

2. AKR/substrain resistance. The in vitro studies will be extended in order to define the antigenic differences between the AKR/J and AKR/Cu cells which are responsible for eliciting in vivo resistance. Backcross and serological studies will be performed in order to discover whether the suspected minor histocompatibility antigen is in fact linked to the Thy locus.

Hybrids of AKR/JxAKR/Cu and AKR/JxCBA/J are being prepared in order to determine whether the F_1 state will confer resistance to the high spontaneous incidence of leukemia observed in AKR/J and AKR/Cu mice. Preliminary studies show that hybrids of AKRxCBA have a longer latent period and lower incidence of leukemia than AKR/CuxAKR/J F_1 mice.

3. AKR/Cu and AKR/J mice will be challenged with the reciprocal lymphoma cells and observed for long-term survival. It will be of considerable interest to determine whether by priming against the reciprocal strain, one can prevent or reduce the strain's own high spontaneous incidence of lymphoma.

Publications

Schmitt-Verhulst, A.-M. and Zatz, M. M.: F_1 resistance to AKR lymphoma cells in vivo and in vitro. J. Immunol. 118: 330-354, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05054-02 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mouse Macrophage Activation with BCG: Relationship to Anti-Tumor Activity In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI W. D. Terry Associate Director for Immunology I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adherent peritoneal cells contain a minor subpopulation of B cells that share some characteristics with macrophages which are non-phagocytic. These cells have potent anti-tumor effect and may be responsible for much of the anti-tumor activity usually ascribed to macrophages.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Previous studies showed that treatment with a variety of agents, thought to activate macrophages promoted resistance to tumors in vivo, in correlation with increased anti-tumor activity of "macrophage" populations taken from such animals and studied in vitro. Other studies have demonstrated a wide variety of functional, morphological, metabolic and surface alterations of macrophages after "activation" by different means. There is little information, however, to indicate which, if any, of the known features of "activation" may be relevant to the anti-tumor activity of activated macrophages, nor even which cells among the population called "macrophages" may be most important for anti-tumor activity. Understanding of these relationships has been hampered by a lack of sufficiently quantitative assays for various macrophage functions. The objectives of the study were to develop in vitro methods for the assay of anti-tumor activity and for certain other macrophage functions, such as phagocytosis, and then to identify relationships between activation and anti-tumor activity which might point to the existence of subsets of cells among "macrophage" populations.

Methods Employed: Mice were injected with BCG, their peritoneal cells collected, and the adherent or nonadherent cells allowed to interact with a variety of mouse lymphoma or fibrosarcoma tumors in microcultures. Inhibition of tumor cell proliferation was assessed by uptake of tritiated thymidine. Assays for the phagocytosis of a variety of particles were either modified or newly developed to allow quantitation under optimal dose-time conditions, rarely attained in previous studies. These particles included radiolabelled starch granules and bacteria, graphite dust and latex beads. Peritoneal cell populations were also studied for a variety of histochemical and surface markers, such as surface immunoglobulin, complement receptors and Fc receptors. Mice treated with a variety of other agents were also studied. Glucose metabolism and the roles of hydrogen peroxide and 2-mercaptoethanol in macrophage-lymphoma cell interaction were examined. Finally, a means was devised for the physical separation of certain subsets of adherent peritoneal cells.

Major Findings: Using a variety of particles, a phagocytic defect was identified in adherent peritoneal cells from BCG treated mice. This appeared to have two components: an increased number of non-phagocytic adherent cells among the peritoneal cells of such mice, and a decrease in the uptake of particles by the remainder of the adherent cells. The non-phagocytic adherent cells were isolated and shown to have marked anti-tumor activity which could not be attributed to the isolation technique. The activity of this subpopulation was such as possibly to account for all the anti-tumor activity of the total adherent cell population. Marker studies suggested that the non-phagocytic adherent cells represent a newly identified subpopulation with some properties of macrophages and others of B lymphocytes.

Significance to Biomedical Research and the Program of the Institute: An unexpected heterogeneity among adherent peritoneal cells was revealed. Strong support was given to the hypothesis that anti-tumor activity of "macrophage" populations used here may in fact reside with a small but potent sub-population, which may not even be macrophages. This would place in a different light the question of the relationship of various tests of macrophage function (such as phagocytosis) to the anti-tumor activity usually attributed to macrophages. Thus, understanding may be fostered in two directions: concerning the basic biology of host defense against tumors, and concerning the tests with which to monitor the effect of immunotherapeutic agents, such as BCG, on host lymphoid-mononuclear cell function.

Proposed Course: This project has now been terminated.

Publications

Nathan, C.F., Asofsky, R., and Terry, W.D.: Characterization of the nonphagocytic adherent cell from the peritoneal cavity of normal and BCG-treated mice. J. Immunol. (in press).

Nathan, C.F., Hill, V.M., and Terry, W.D.: Isolation of a sub-population of adherent peritoneal cells with anti-tumor activity. Nature 260: 146-148, 1976.

Nathan, C.F. and Terry, W.D.: Decreased phagocytosis by peritoneal macrophages from BCG-treated mice - Induction of the phagocytic defect in normal macrophages with BCG in vitro. Cell. Immunol. 29: 295-311, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05055-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulatory Influences on Cell-Mediated Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. M. Nadler	Clinical Associate	I NCI
OTHER:	R. J. Hodes	Head, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Investigation was carried out on the regulatory mechanisms generated in mixed lymphocyte reactions (MLR). Suppressor cells were found to be generated by in vivo or in vitro priming to alloantigen (or by in vitro culture in the absence of alloantigen). The suppressor cell populations generated in vivo and in vitro were compared in MLR and cell-mediated lympholysis (CML) for antigen specificity, radiation sensitivity, mitomycin sensitivity, T cell dependence and ability to suppress via a soluble factor. These comparisons were carried out to permit analysis of regulatory influences which may be involved in the generation of allograft responses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: In addition to the effector cells generated in response to antigen stimulus, there has been increasing data presented to suggest that cell-mediated regulatory influences are also activated by antigenic stimulus. It has been the object of these studies to define the regulating influence governing the generation of T cell-mediated responses.

Methods Employed: In vivo priming to alloantigen was adapted from the methods of Rich and Rich. Both the in vitro and in vivo generated suppressor populations were tested in either MLR and/or CML for their regulatory effects on these responses. These suppressive populations were further characterized by: 1) their sensitivity to irradiation or mitogen; 2) T cell dependence; 3) the ability of soluble cell-derived factors to mediate suppression; and 4) antigen specificity of suppression.

Major Findings: I. Comparison of Culture-Induced and Alloantigens Induced Suppressor Cells.

Suppressor cells generated by an in vitro culture of spleen cells were compared with suppressor cells generated by in vivo priming with alloantigen. Suppressor cells produced by in vitro culture were themselves unable to respond in CML or MLR; were able to actively suppress the CML and MLR responses of untreated responding cells; were mitomycin sensitive, and produced no easily demonstrable suppressive supernatant. Alloantigen-primed cells were able to respond in CML and MLR; could suppress proliferation in MLR, but were able to suppress CML only after mitomycin treatment; and, produced suppressive supernatant active in suppressing both CML and MLR. These differences suggest that the two suppressor populations may be distinct suppressor populations or at least different differentiation stages of the same lineage.

II. Antigen Specific and Nonspecific Suppressor Activity Generated During MLC.

Antigen-specific and non-specific suppressor cell activities produced during in vitro MLC were studied. Antigen-nonspecific suppression was active in suppressing CML sensitization to alloantigens including those to which the suppressor population had not been previously exposed. This form of suppression was radiation sensitive and T cell dependent. In contrast, antigen-specific suppression affect only those CML sensitizations in which the relevant sensitizing antigens either 1) was the same antigen to which the suppressor populations was previously primed, or 2) was presented on the same cell as that alloantigen. Antigen specific suppression was resistant to irradiation and was also T cell dependent. These data suggest active mechanisms which serve to control the antigen specificity of primary and secondary responses to alloantigens.

Significance to Biomedical Research and the Program of the Institute: States of in vivo tolerance or immune hyporesponsiveness have been associated with active suppressive populations in a number of systems. In addition, the possible role of speicfic or nonspecific immune suppression in the evaluation or progression of malignancy has been suggested. The studies described above may provide information concerning the mechanism of such in vivo immunosuppression and possible means of modifying the suppression as a therapeutic manipulation of the immune response.

Proposed Course of Project: Further studies intended to characterize effector and regulatory cell populations are in progress. The initial approaches have included analysis of cell surface alloantigens, including Ia and Ly, on these populations. By these methods we hope to characterize the regulatory populations which influence the generation of cell-mediated cytotoxic responses to transplantation antigens.

Publications

Nadler, L. M. and Hodes, R. J.: Regulatory mechanisms in cell-mediated immune responses. II. Comparison of culture-induced and alloantigen-induced suppressor cells in MLR and CML. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05056-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Specific Removal of Histocompatibility Antigen-Reactive Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Neefe, Jr.	Investigator	I NCI
OTHER:	D. H. Sachs	Head, Transplantation Biology Section	I NCI

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.8	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Receptors for transplantation antigens may be visualized directly on unsensitized cells with the use of an anti-idiotypic antisera. It should be possible to eliminate these cells specifically by adherence to a cellular immunoadsorbent or by treatment of the unsensitized population with sera directed at the specific binding site. Alternatively, active and continuing control of the cells reactive to specific transplantation antigens may be possible with suppressor cells specific for the binding site. Immunoadsorption using spleen cell monolayers is highly efficient and specific in removal of cytotoxic cells from a sensitized population but is not effective in removing precursors of these cells. Antisera with the capacity to inhibit in vitro generation of cytotoxic cells have been raised. To date, activity of these sera has been too low to permit clear conclusions about specificity of the inhibition. Suppressor cells have also been generated. These cells have the property of inhibiting the in vitro generation of cytotoxic cells and in some experiments appear to be specific. Clear interpretation has been hindered by the substantial nonspecific augmenting properties these suppressor populations also possess.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Several lines of evidence indicate clonal diversity of T lymphocytes reactive to transplantation antigens and recently it has been possible to visualize directly receptor-bearing lymphocytes in unsensitized populations. If precursors of cells reactive to transplantation antigens bear receptors to those antigens, it should be possible to render a lymphoid population tolerant of those antigens either (1) by adherence of the receptor-bearing cells to a solid-phase immunoadsorbent bearing the antigens or (2) by inhibition of the receptor-bearing cells using antibody with specificity for the binding site of the receptor (anti-idiotypic antibody). If precursors of cells reactive to transplantation antigens do not all bear receptors for those antigens or if some precursors bear labile receptors or receptors in low density, the above approaches may fail. In that case, active and continuing control of the generation of effector cells may be achieved through the use of suppressor cells. If such cells are specific, the specificity could be directed at the binding site of the receptors of the effector cells and their precursors. All three approaches have been investigated.

In addition to the obvious usefulness of such techniques and principles for study of the generation of cellular immunity, some clinical applications are of importance, as in specific tumor immunotherapy. Allogeneic cells sensitized by whatever means against tumor-associated antigens could prevent the appearance or recurrence of tumor, but they must also be rendered incapable of causing graft-vs-host disease by elimination of any capacity to react to the transplantation antigens of the host.

Methods Employed: Studies were performed using normal mouse spleen cells. Reactivity to transplantation antigens was assessed at the effector stage by the capacity to generate specifically cytotoxic cells in mixed lymphocyte culture. Cellular immunoadsorbents were monolayers of normal spleen cells attached to polystyrene coated with poly-L-lysine. Anti-idiotypic antibodies were generated by multiple immunization of F1 hybrid mice with cytotoxic cells of one parent strain sensitized to transplantation antigens of the other parent. Putative suppressor cells were similarly generated by in vitro "sensitization" of F1 hybrid cells to cytotoxic cells of one parent.

Major Findings: Spleen cell monolayers removed cytotoxic cells from a previously sensitized population efficiently and specifically. Apparent absorption was not caused by release of monolayer cells to serve as competitive inhibitors in the cytotoxic assay. In contrast, unsensitized cells could not be absorbed, and, in fact, prior exposure of an unsensitized population to a monolayer of any antigenic constitution nonspecifically increased the activity provoked by specific sensitization. Absorption of unsensitized cells on fibroblast monolayers yielded an identical result.

Since in vitro sensitization drives cells to the more adsorbable cytotoxic state, it was thought that precursors might also become more adsorbable after

sensitization of the population containing them. Therefore, cytotoxic cells at or after the peak of activity were absorbed and cytotoxic activity was reduced or removed. After a subsequent secondary boost in vitro those cells showed not a decrease but a very substantial increase in activity. This augmented activity arose in a fashion independent of the antigenic constitution of the monolayer. Mixing experiments designed to show adherence of nonspecific suppressors to the monolayer did not confirm the presence of such suppressors. A second absorption of these cells of augmented cytotoxic activity diminished their activity; when these populations were sensitized in vitro for a third time the original reactivity was more than reconstituted.

These experiments suggest that precursors of cytotoxic cells are not readily removed by adherence to a spleen cell or fibroblast monolayer because their receptors either are labile and easily released or are sparsely represented on the cell surface.

Anti-idiotypic antibody has been raised and tested for its ability to inhibit generation of specific cytotoxic cells in vitro. These sera were inhibitory, but further investigation has been hampered because the activity is present in low titer. An additional problem is that preimmune sera inhibited, although to a lesser extent.

Suppressor cells with the capacity to inhibit generation of cytotoxic responses in vitro have been raised. In some experiments this suppression has appeared to be specific. If confirmed, this specificity could be for allotypic determinants of the responding cell or for the binding site of the responder's receptors. Unequivocal interpretation of suppression and its specificity has been hindered by the substantial augmentation of cytotoxic activity observed upon addition of the suppressive population to some control sensitization mixtures in which specific suppression was not expected.

Significance to Biomedical Research and the Program of the Institute: Transplantation may be useful for cancer therapy in several settings. Transplants of lymphoid cells appropriately sensitized may control the appearance or growth of tumors. Transplants of organs may replace organs ravaged by tumors. Rescue with transplants of bone marrow may permit use of doses of anticancer chemotherapy which would otherwise be lethal. In each setting, success depends in part on specific control of responses to histocompatibility antigens. This project examines one means for eliminating these responses specifically.

Proposed Course of Project: Since receptors are demonstrable on precursors of cells reactive to transplantation antigens, efforts will be continued to remove or control the receptor-bearing cells by specific means. The heterogeneity of binding sites used to stimulate anti-idiotypic sera or cells will be restricted by use of F1's of strains with only very small histocompatibility differences. The intensity of the "immunogenic" stimulus will be increased by utilizing populations of cytotoxic cells with a greater proportion of receptor-bearing cells. The possibility that cytotoxic cells as "immunogen"

kill the anti-idiotypic precursor they are intended to stimulate will be eliminated by using fixed cytotoxic cells or unsensitized populations as a source of receptor-bearing cells.

Publications

Neefe, J. R., and Sachs, D. H.: Specific elimination of cytotoxic effector cells. I. Adsorptive behavior of effectors and their precursors on spleen cell monolayers. J. Exp. Med. 144: 996-1008, 1976.

Neefe, J. R., and Sachs, D. H.: Specific elimination of cytotoxic cells. II. Reduction of cytotoxic activity by adsorption on monolayers results from removal of cytotoxic cells and not from inhibition of their activity. Cell. Immunol. 29: 129-136, 1977.

Neefe, J. R., and Sachs, D. H.: Adherence properties of cytotoxic cells and their precursors on spleen cell monolayers. Transplant. Proc. 9: 797-799, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05058-02 I

PERIOD COVERED

Judy 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunoregulation by Antibody

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Pincus	Investigator	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI
	C. O. Cowing	IPA Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Ig subclass specificity of immunoregulation by Ab will be examined with the use of subclass purified Ab. The fine specificity of Fc receptors on immunocompetent cells will be determined in an effort to assess the role of these receptors in activation of the cells by antigen and control of the immune response. These studies will include both antibody raised against protein antigens (including synthetic polypeptides) and anti-idiotype antibodies. Antibodies to Keyhole Limpet Hemocyanin and (T,G)-A-L have been raised. The antibody to (T,G)-A-L has been affinity purified and used for immunization in an attempt to raise anti-idiotype antibodies. A radioimmunoassay for detection of the latter has been developed. A T-cell antigen-specific, macrophage dependent, proliferative assay has been developed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) To examine immunoregulation by antibody purified into class and subclass of Ig; (2) To determine the subclass specificity of Fc receptors on murine immunocompetent cells and; (3) To determine the role, if any, of Fc receptors in the induction or suppression of the immune response.

Methods Employed: (1) Immunization of responder and non-responder mouse strains with (T,G)-A-L by various protocols. (2) Purification of Ab into class and subclass by iso-electric focusing and solid phase immunoadsorption using heavy chain specific antisera. (3) Use of Ag-Ab complexes and indirect immunofluorescence on purified cell populations. (4) In vitro and in vivo assays for the response of the cells to antigen: proliferation, Ab production, CMI, helper function, suppressor activity. (5) Production of anti-idiotypic antisera in both syngeneic and xenogeneic animals.

Major Findings: 1) A very large quantity of anti-TGAL and anti-KLH Ab has been raised in ascites form. This ascites when measured using a radioimmunoassay contains a high titer of specific antibody. The anti-TGAL antibody has been purified on solid phase immunoadsorbants, yielding a single IgG line on immunoelectrophoresis. Lewis rats are being immunized with this product in an attempt to produce anti-idiotypic sera. 2) A radioimmunoassay has been developed that is able to detect and titer Ab down to the nanogram level. This assay has been applied to the following protein antigens: TGAL, KLH, Staphylococcal Nuclease and IgG. In addition inhibition of the RIA has been used to detect anti-idiotypic antibodies. 3) A T-cell proliferative assay has been developed using murine lymph node cells. This assay is macrophage dependent, and the macrophages can be pulsed with Ag prior to culture, thus obviating the need for Ag to be present continuously during the culture period.

Significance to Biomedical Research and the Program of the Institute: Characterization of the conditions under which Ab stimulates or suppresses either CMI or Ab production would increase the potential for control of the immune response. Determination of the fine specificity and function of Fc receptors on immunocompetent cells would clarify the mechanisms of antibody dependent cell-mediated cytotoxicity and of Ab-dependent tumor cell enhancement, i.e., immune mechanisms which may play a role in the control or lack thereof of malignant cells.

Proposed Course of Project: 1) To clarify the relationship between the Ag presenting cells (most likely an Fc^+, Ia^+ macrophage) and T cells in the proliferation assay. 2) To study the effects of Ab and Ag-Ab complexes on this relationship and to see if the Fc receptor plays a role in these effects. In addition the subclass specificity of this effect will be evaluated. 3) To extend any findings from the above experiments to other in vivo and in vitro assay systems. 4) To evaluate the role of anti-idiotypic Ab's in the control of the immune response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05059-02 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Antigen Specific T-Cell Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Clinical Associate	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

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0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antigen specific T-cell factors are most likely the solubilized T-cell antigen receptor, since it is unlikely that the T-cell has two different antigen specific molecules, the factor and a different receptor. Therefore, antigen specific T-cell factors offer a direct way to study the structure of the T-cell antigen receptor. In addition, the immunoregulatory functions of these factors provide a way of studying specific B-cell activation and T-B cooperation.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The three major objectives of this project are: (1) the confirmation of the existence of antigen specific T-cell factors, (2) the physical characterization of such factors, and (3) the study of the immunoregulatory function of antigen specific T-cell factors in T-B and T-T cooperation.

Methods Employed: Thymocytes from C57BL/10 mice educated to TGAL are cultured with TGAL for 6-8 hours under serum-free conditions. The supernatants from these cultures contain an antigen specific T-cell product which can replace T-cells in an adoptive transfer experiment. The biologic activity of this factor is assayed by adoptively transferring into a syngeneic animal either bone marrow cells and TGAL, bone marrow cells plus educated thymocytes and TGAL, or bone marrow cells plus the supernatant factor and TGAL. The development of specific antibody producing cells in the spleens of the transferred animals is assayed by the Jerne plaque technique. The following methods are used to characterize the factor:

(a) the use of genetically different strains of mice to produce and respond to the factor,
(b) column purification of the factor,
(c) titration of varying amounts of factor with B-cells,
(d) gel filtration to determine molecular weight,
(e) absorption of factor by anti-Ia, antigen, anti-immunoglobulin, and anti-idiotype immunoadsorbents.

Major Findings: TGAL specific T-cell factor can be obtained from educated thymocytes cultured with TGAL under serum-free conditions for 6-8 hours. This factor can cooperate with normal bone marrow cells and TGAL to induce the development of cells which secrete specific anti-TGAL antibody in the absence of T cells. The factor produced by TGAL educated thymocytes is antigen specific and does not cooperate with B-cells to form anti-SRBC antibodies.

Significance to Biomedical Research and the Program of the Institute: T-cells are one type of lymphoid cell involved in tumor recognition and destruction, yet the T-cell antigen recognition system is poorly understood. Greater insight into the structure and function of the T-cell antigen receptor will likely lead to a deeper understanding of the roles T-cells and their products play in tumor immunology. Furthermore, antigen specific T-cell factors can function as immunoregulatory molecules either enhancing or suppressing antigen specific immune responses. They offer a potentially potent tool for manipulating the immune response against specific tumor targets.

Proposed Course of Project: Further work on this project will begin when a suitable in vitro system is available for assay of the factor. Assuming that this is developed (good progress has been made to date) then the project will be directed toward developing an antigen specific T-cell factor against Staphylococcal Nuclease. The immune response to Staphylococcal Nuclease is also under Ir gene control and appears to be controlled by the Ir-b subregion.

We then hope to determine several structural and functional characteristics of this factor. Structurally, we hope to determine (a) whether the antigen specific T-cell factor bears the same idiotype as immunoglobulin (the antigen specific B-cell product), (b) if the Ia antigens constitute a portion of the factor, and (c) what molecules bind the factor to the B-cell surface, and specifically whether they are related to either the Ia antigens and/or the Fc receptor. Functionally, we would like to determine (a) whether the factor, in high concentration, will lose its antigen specificity and become nonspecific, and (b) if the antigen specific T-cell enhancing and suppressing factors are identical but reflect different biologic functions of the factor under different conditions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 5060-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Physiological Role of Lymphocyte Surface Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Henkart	Senior Investigator	I NCI
OTHER:	J. L. Ryan	Investigator	I NCI
	M. Shinitzky	Visiting Scientist	I NCI
	S. Ben-Sasson	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracellular levels of cAMP and cGMP were measured by radioimmunoassay in mouse spleen cells settled onto surfaces coated by antigen-antibody complexes or $F(ab')_2$ complexes in the presence or absence of a mitogenic concentration of LPS. cAMP levels were consistently elevated (1.5x) after 30 minutes of contact with immobilized complexes; however, $F(ab')_2$ complexes did not induce this change, nor did LPS affect the cAMP levels. No significant change in cGMP levels was found to be induced by immobilized complexes.

Some preparations of gangliosides were found to be mitogenic for mouse spleen cells. Purified ganglioside fractions which were non-immunogenic inhibited LPS-induced mitogenesis but not PHA induced mitogenesis.

MLC type reactions between lymphocytes and tumor cells were shown to be enhanced by inclusion of dextran and polyethyleneglycol in the culture medium. These results were interpreted as being due to enhanced lymphocyte-tumor interaction via specific receptors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To define the physiological function of the Fc receptor. To test the hypothesis that lymphocyte glycolipids may play a physiological role in the immune response. To attempt to enhance weak lymphocyte-tumor cell interactions.

Methods Employed: Mouse spleen lymphocytes were allowed to settle on petri plates coated with TNP protein with and without anti TNP. After various times the supernatant and cells were collected, treated with TCA and the cyclic nucleotide levels measured by radioimmunoassay after separation by ion exchange chromatography. Gangliosides from bovine brain were purchased and further fractionated on TLC. Standard culture conditions were employed for MLC and mitogenesis experiments.

Major Findings: Intracellular levels of cAMP and cCMP were measured by radioimmunoassay in mouse spleen cells settled onto surfaces coated by antigen-antibody complexes of $F(ab')_2$ complexes in the presence or absence of a mitogenic concentration of LPS. cAMP levels were consistently elevated (1.5x) after 30 minutes of contact with immobilized complexes; however, $F(ab')_2$ complexes did not induce this change, nor did LPS affect the cAMP levels. No significant change in cGMP levels were found to be induced by immobilized complexes.

The commercial ganglioside preparations which are mitogenically active were further fractionated by precipitation. The active fraction is less chloroform soluble than most gangliosides. It contains protein and sugar and therefore the activity may be a contaminant rather than a glycolipid. The specifically suppressive ganglioside fractions appear to coincide with the major classes of gangliosides in cells. It is hypothesized that these gangliosides may compete with LPS for binding sites in the membrane.

Enhanced thymidine incorporation due to an MLC type reaction was observed when the hydrophilic polymers dextran and PEG were present in the medium during culture of lymphocytes and syngeneic tumor cells. Similar enhancement was observed for classical MLC reactions done at suboptimal conditions. The polymers alone are not mitogenic.

Significance to Biomedical Research and the Program of the Institute: The increased cAMP levels mediated by lymphocyte Fc receptors confirms our proposal on the mechanism of the inhibitory effects previously observed. The ganglioside experiments provide evidence that naturally occurring cell surface molecules can modulate the immune response. Finally enhanced in vitro responsiveness of lymphocytes to tumor cells may be important in developing techniques for enhancing immunity to tumors.

Proposed Course of Project: The mitogenic activity of ganglioside preparations is being further purified to identify the chemical nature of the active material. Further experiments are being carried out to identify the mechanism of the LPS mitogenic inhibitory activity of gangliosides.

Publications

Ryan, J.L. and Henkart, P.A.: Fc receptor mediated inhibition of B-lymphocyte activation. J. Exp. Med. 144: 768-775, 1976.

Ryan, J.L. and Henkart, P.A.: Inhibition of B lymphocyte activation by interaction with Fc receptors. Immunol. Commun. 5: 455-468, 1976.

Ben-Sasson, S. and Henkart, P.A.: Enhancement of weak MLC type reactions by hydrophilic polymers. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05061-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Specific Lymphocyte Adhesion to Target Surfaces

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. A. Henkart	Senior Investigator	I NCI
OTHER:	E. L. Alexander	Guest Worker	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously studied the adhesion of human lymphocytes to surfaces coated with antigen-antibody complexes as a means of studying the killer-target cell interaction during antibody dependent lymphocyte mediated cytotoxicity and as a means of separating cells on the basis of their Fc receptors. We have extended this work to studying the adhesion of human B cells via sIg, using goat anti-human Ig bound to plastic surfaces. Such adhesion can be used to isolate B lymphocytes, leaving lymphocytes bearing high affinity Fc receptors unbound. Tissue culture flasks coated with these ligands can preparatively remove FcR positive sIg negative cells or sIg positive FcR negative B cells; 25×10^6 cells can be processed per flask. These results confirm previous data that B cells do not have avidly binding Fc receptors.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Achieve specific separation of lymphocyte populations via cell surface receptors.

Methods Employed: Affinity purified goat anti-human Ig adsorbs to plastic surfaces from solution. After washing, lymphocytes in Eagle's medium are allowed to settle onto the surface. After a short incubation, the unbound cells are washed away.

Major Findings: We have previously studied the adhesion of human lymphocytes to surfaces coated with antigen-antibody complexes as a means of studying the killer-target cell interaction during antibody-dependent lymphocyte-mediated cytotoxicity and as a means of separating cells on the basis of their Fc receptors. We have extended this work to studying the adhesion of human B cells via sIg, using goat anti-human Ig bound to plastic surfaces. Such adhesion can be used to isolate B lymphocytes, leaving lymphocytes bearing high affinity Fc receptors unbound. Tissue culture flasks coated with these ligands can preparatively remove FcR positive sIg negative cells or sIg positive FcR negative B cells; 25×10^6 cells can be processed per flask. These results confirm previous data that B cells do not have avidly binding Fc receptors.

Significance to Biomedical Research and the Program of the Institute: Cell separation techniques such as these enable manipulations to be carried out which can dissect the immune response in vitro.

Proposed Course of Project: These experiments have completed this project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05062-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Application of Rapid Flow Microfluorometry to Cell Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

			IB NCI
PI:	J. R. Wunderlich	Senior Investigator	IB NCI
OTHER:	W. E. Paul, M.D.	Chief, Lab. of Immunology	LI NIAID
	T. M. Chused, M.D.	Medical Officer (Research)	LMI NIDR
	D. H. Sachs, M.D.	Head, Transplantation Biology Section	IB NCI
	H. B. Dickler, M.D.	Senior Investigator	IB NCI
	P. A. Henkart, Ph.D.	Senior Investigator	IB NCI
	R. H. Schwartz	Investigator	LI NIAID
	J. N. Weinstein	Investigator	LTB, NCI
	H. C. Morse	Senior Investigator	LMI NIAID
	J. N. Hartley	Senior Investigator	LVD, NIAID

COOPERATING UNITS (if any)

CDR Irwin Scher, M.D., Aftab Ahmed, Ph.D., Dept. of Clinical and Experimental Immunology, Naval Medical Research Institute, Bethesda, MD. Graham L. Campbell, Ph.D., The Wistar Institute of Anatomy and Biology, Philadelphia, PA.

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 years	3 1/2	1 1/2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using rapidflow microfluorometry for analysis and sorting of cells, aspects of the following projects have been supported during the previous years: 1) further characterization of a subclass of B lymphocytes which have a critical role in humoral immune responses; 2) expression of Ia antigens on macrophages, B lymphocytes and thymocytes; 3) characteristics of liposome-cell interactions; 4) expression of cell surface antigens coded for by xenotropic murine leukemia virus.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this project is to utilize rapid flow microfluorometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS), Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project, emphasizing those aspects most heavily supported by use of rapid flow microfluorometry (FMF).

Subclasses of murine B cells have been further defined through use of FMF in cooperation with Dr. Scher and colleagues. Earlier a subclass of cells had been identified as a population of mature B cells necessary for normal immune responses to T independent antigens and B cell mitogens. These cells, which characteristically express intermediate levels of total surface immunoglobulin, represent about 25% of B cells found in normal adult spleens. Using the level of surface immunoglobulin (SIg) expression as a marker for these B cells, more recent studies have shown that these cells develop during the first 4 weeks of life, that their maturation is controlled in part by the x-chromosome, that they bear complement receptors and surface antigens necessary for M-locus defined mixed lymphocyte reactions and that they have low levels of surface IgM; surface IgG and IgA are undetectable. IgD is suspected to comprise a large percentage of the total SIg on these cells. In addition, an antiserum has been produced and characterized which reacts preferentially with this subclass of B cells by recognition of a B-cell differentiation antigen designated Lyb 5.1. Finally, this subclass has been further separated on the basis of quantity of SIg. Cells with high intermediate amount of SIg bear complement receptors but not M-locus product, whereas cells with low intermediate SIg bear both.

Expression of Ia antigens, products of a genetic region controlling immune responses to thymus dependent antigens, has been studied in cooperation with Drs. Dickler, Sachs, B. Schwartz and R. Schwartz. Murine thymocytes and peritoneal macrophages express Ia antigens on their surfaces. These products, detected by specific anti-Ia antisera, are present at distinctly lower concentrations (1-15 to 1-50) than found on murine B-lymphocytes. Most likely, only a subpopulation (8-15%) of peritoneal macrophages express Ia antigens. Additional studies using surface antigen capping techniques and quantitative analysis of remaining surface markers indicate that Ia antigens and Fc receptors on B lymphocytes are not identical and for the most part do not lie in close proximity to each other when expressed in the native state.

Liposome-cell interactions, reactions of interest as a means for introducing membrane impermeable substances into cells, are being studied in collaboration with Drs. Henkart, Weinstein, and Blumenthal. In contrast to

conventional radioisotope methods for studying these reactions, results of rapid flow microfluorometry have shown that the location of fluorescent substances can be identified, whether still within the vesicles, within the cells, or within the extracellular fluid. Quantitative evaluation of these parameters has permitted accurate stochiometric analysis of liposome-cell fusion and cell uptake and loss of fluorescent substances. Moreover, microfluorometry analyzes single cells and distinguishes between live and dead cells--capabilities which have allowed analysis of population statistics of the process, correcting for the presence of dead cells.

Expression of cell surface antigens coded for by xenotropic murine leukemia virus (XenCSA) on normal lymphocytes and malignant cells is being studied with Drs. Morse, Chused, Mathieson, and Hartley. Results have shown that XenCSA are preferentially expressed in certain strains of mice and on certain subpopulations of normal cells within these strains. Expression of XenCSA differs quantitatively between positive strains. The presence of XenCSA does not depend on the production of infectious viral particles. Qualitative expression of XenCSA within a mouse strain is usually associated with the expression of GIX viral antigens; yet XenCSA are distinct from the GIX antigen(s). Analysis of recombinant inbred lines between C57BL/6J and DBA/2J, and parent times F_1 backcrosses has shown that expression of XenCSA is controlled in part by a gene on chromosome 4 (linkage group 7), which is linked to the Fv-1 locus which controls susceptibility of cells to infection by ecotropic viruses. Also of interest is the finding that lymphocytes from all mouse strains with a high incidence of autoimmune disease, so far examined, express XenCSA.

Significance to Biomedical Research and the Program of the Institute;
Investigating the nature of B lymphocytes and Ia antigens is important to tumor immunology insofar as both of these elements have critical roles in antitumor immune responses. B lymphocytes as an obligatory link in the humoral immune response, may contribute to immune rejection of leukemia cells, are required for antibody dependent cellular cytotoxicity and may block T cell mediated cytotoxicity. Ia antigens on the other hand are products of a genetic region which controls immune responses to T dependent antigens among which are tumor associated antigens.

Understanding the process of liposome-cell interactions is important because this technique is among the most promising means for introducing membrane impermeable substances into cells and as such is important for developmental chemotherapy and experimental cell transformation.

Identification of xenotropic viral antigens on subclasses of normal lymphoid cells has important implications regarding normal host regulation of these cells, for malignant transformation of normal cells by murine leukemia viruses and finally for the specificity of reagent antisera currently used in tumor immunobiology, many of which may unsuspectingly identify cells bearing surface viral antigens.

Proposed Course of Project: As in the past, rapid flow microfluorometry will be used for selected, appropriate projects.

Publications

Scher, I., Ahmed, A., Sharrow, S. O., Steinberg, A. D. and Paul, W. E.: Genetic control of B-lymphocyte function in the CBA/N mouse strain: A model for examining the mechanism of B-lymphocyte activation. In Oppenheim, J. and Rosenstreich, D. (Eds.): Mitogens in Immunobiology. New York, Academic Press, 1976, pp. 325-335.

Scher, I., Sharrow, S. O., Wistar, R., and Paul, W. E.: Density of surface Ig on B cells: Ontogenic studies of a population of splenic B cells in normal mice and mice with an X-linked B-lymphocyte immune defect. In Eijsvoogel, V. P., Roos, D., and Zeijlemaker, W. P. (Eds.): Leukocyte Membrane Determinants Regulating Immune Reactivity. New York, Academic Press, 1976, pp. 189-195.

Scher, I., Sharrow, S. O., and Wistar, R., Jr.: B lymphocyte heterogeneity: ontogenetic development and organ distribution of B-lymphocyte populations defined by their density of surface immunoglobulin. J. Exp. Med. 144: 494-506, 1976.

Scher, I., Sharrow, S. O., and Paul, W. D.: X-linked B-lymphocyte populations defined by their density of surface immunoglobulin. J. Exp. Med. 144: 507-518, 1976.

Schwartz, R. H., Dickler, H. B., Sachs, D. H., and Schwartz, B. D.: Studies of Ia antigens on murine peritoneal macrophages. Scand. J. Immunol. 5: 731-743, 1976.

Ahmed, A., Scher, I., Sharrow, S. O., Smith, A. W., Paul, W. E., Sachs, D. H., and Sell, K. W.: B-lymphocyte heterogeneity: Development and characterization of an alloantiserum which distinuishes B-lymphocyte differentiation alloantigens. J. Exp. Med. 145: 101-109, 1977.

Weinstein, J. W., Yoshikami, S., Henkart, P., Blumenthal, R., and Hagins, W. A.: Liposome-cell interaction: Transfer and intracellular release of a trapped fluorescent marker. Science 195: 489-492, 1977.

Campbell, G. LeM., Schachner, M., and Sharrow, S. O.: Isolation of glial cell-enriched and depleted populations from mouse cerebellum by density gradient centrifugation and electronic cell sorting. Brain Res., in press.

Schwartz, B. D., Kask, A. M., Sharrow, S. O., David, C. S., and Schwartz, R. H.: Partial characterization of Ia antigens from murine thymocytes. Proc. Natl. Acad. Sci., in press.

Scher, I., Ahmed, A., Sharrow, S., and Sell, K. W.: The murine minor lymphocyte stimulating locus defines a B cell subclass whose expression is dependent on genes on the X chromosome. Transplant. Rev., in press.

Dickler, H. B., Kubicek, M. T., Arbeit, R. D., and Sharrow, S. O.: The association between Ia antigens and Fc receptors on murine B lymphocytes is probably ligand-induced. Proc. III Ir Gene Workshop, in press.

Scher, I., Ahmed, A., Sharrow, S. O., and Paul, W. E.: Maturation of murine B lymphocytes. In Oppenheim, J. and Rosenstreich, D., (eds.): Development of Immunological Host Defense Mechanisms, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05063-01 I

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Idiotype Specific T-Cell Responder

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lee D. Leserman	Clinical Associate	I NCI
OTHER:	William D. Terry	Associate Director for Immunology	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro assays for studying specific cell-mediated immunity to plasma cell tumors of mice have been established. Antisera directed against the idiotypic antigens of myeloma proteins produced by these tumors have been produced. These systems will now be used to study T-cell recognition of idiotypic antigens.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Lymphoid cells of mice can be sensitized to syngeneic tumor cells in vitro, and will produce cytotoxic effector cells which can kill those tumor cells. Despite this apparent ability to elicit an effective immune response, these tumors will grow and kill a syngeneic host following transplantation with little or no evidence of an immune response against them.

Experiments which purport to study responses to tumor specific antigens are difficult to interpret because of the problem of reaction to viral antigens or derepressed fetal antigens, perhaps not unique to the tumor or relevant to tumor antigens present in vivo.

In order to follow the response to antigens known to be present on and produced by tumors, we study the response of BALB/c mice to myeloma tumors of BALB origin. It is known that these tumors bear idiotypic determinants which will elicit T-helper and B-cell responses.

Long range goals of this project are:
(1) to find whether cytotoxic T-cells recognize idiotypic determinants; (2) to see whether immunologic manipulations can increase the representation of cells sensitive to these idiotypes; (3) to determine if these manipulations can induce protective responses in the host or in vitro.

Methods Employed: Cell-mediated immunity is measure by release of ^{51}Cr from target cells incubated in vitro with sensitized lymphoid cells. These cells may be sensitized by immunization with tumor or idiotypic determinants, or by passive immunization with anti-idiotypic antibody. We also will measure effects on idiotype recognition by immunizing with antigens which are bound by idiotypes of known hapten-binding specificity.

Major Findings: (1) Plasma cell tumors have been shown to be excellent targets for immune lysis in that they lyse easily in the presence of specific immune cells and have acceptably low background ^{51}Cr release. These tumor cells can be killed by cells not able to lyse other syngeneic tumors, though it is not known whether these killer cells are directed at idiotypic markers. (2) Methods have been standardized to measure very small quantities of idiotype and anti-idiotype present in serum, and on the surfaces of cells. This will enable accurate measurement of host response to idiotypes present on tumor. (3) The ability to measure specific antigens on tumor cells enables a study of selection of mutant tumor surface antigens in response to host immune recognition, which may enable the tumor to evade host attack.

Significance to Biomedical Research and the Program of the Institute: Much current therapy assumes that specific or non-specific enhancement of host mechanisms will promote survival. Despite this assumption, responses to well defined antigens produced by tumors have been poorly characterized. Insight into recognition of self-antigens by lymphoid cells should provide theoretical and practical information concerning regulation of the immune response, autoimmunity and response to tumors.

Proposed Course of Study: Primary effort will be directed at measuring the response of idiotype specific T-cells to tumor antigens and to hapten-modified self.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05064-01 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Clinical Associate	I	NCI
Other:	R. J. Hodes	Head, Immunotherapy Section	I	NCI
	H. B. Dickler	Senior Investigator	I	NCI
	C. Cowing	IPA Investigator	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to more precisely define the nature and function of genes controlling the cellular interactions involved in the antibody response and/or Ir genes controlling response to certain soluble antigens, an in vitro system for generating both primary and secondary antibody responses to soluble antigens has been developed and characterized. Responses in the system are T-cell and macrophage dependent, and for TNP-(T,G)-A--L and TNP-(H,G)-A--L are clearly under H-2 linked Ir gene control.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The three major objectives of this project are: (1) the determination of which cellular interactions are governed by Ir genes so as to determine the method by which these genes function; (2) the characterization of the genetic restrictions on the cellular interaction required for the induction of the antibody response to antigens not under Ir gene control; and (3) the study of the regulation of B-cell function by T cells and macrophages and their soluble products.

Methods Employed: Normal or carrier primed spleen cells are dispersed into single cell suspensions and put into microcultures with TNP modified antigens (KLH, TGAL, HGAL, GAT, OVA, Nase, etc.) for 4 days. The cells are then harvested and assayed for Ig or IgG anti-TNP antibody-producing cells by the slide modification of the Jerne plaque technique.

Splenic B cells are obtained by treating spleen cells with a T-cell specific rabbit antimouse brain serum and complement. Splenic T cells are obtained by passage over a nylon wool column. Splenic macrophages are obtained by allowing spleen cells to adhere to glass petri dishes for 2 hrs and then discarding the nonadherent cells and collecting the adherent cells by the addition of EDTA. Spleen cells are depleted of macrophages by passage over a G10 sephadex column.

Major Findings: A primary response to TNP-modified derivatives of a wide variety of antigens, including the synthetic polypeptides, can be obtained. These in vitro responses are T-cell and macrophage dependent, and for the case of TGAL and HGAL are under H-2-linked Ir gene control.

Significance to Biomedical Research and the Program of the Institute: The study of Ir gene control of the immune response has been limited by a lack of in vitro assays in which Ir gene functions are expressed. The development of such an assay system has been accomplished and provides a major advance in this field. In vitro analysis of Ir gene expression can lead to a greater understanding of how Ir genes function and/or the immune response is regulated. Such understanding will lead to the ability to purposefully regulate immune responses in human disease.

Proposed Course of Project: The project will be directed toward a number of immediate goals: (a) localization to a specific splenic subpopulation of the Ir gene defect in nonresponder mice; (b) elucidation of any genetic restriction on the ability of macrophages, T cells, and B cells to cooperate with one another for the induction of an antibody response; (c) the role of the Ia antigens in cellular interaction, and (d) characterization of antigen-specific T-cell helper and suppressor factors by the use of the in vitro assay.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05065-01 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunologic Function of Macrophages

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. O. Cowing	IPA Investigator	I NCI
OTHER:	H. B. Dickler	Senior Investigator	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.875	0.875	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to explore the mechanism by which macrophages present antigen to lymphoid cells and how they function in the control of the immune response. Recent work under this project has shown that different populations of murine adherent phagocytic cells bear different levels of Ia antigens on their membranes and that this is correlated with their ability to activate T lymphocytes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To evaluate the role of Ia antigens on macrophages in terms of the activation of subsets of B and T lymphocytes. 2) To determine the relationship between macrophage Ia antigens and the expression of Ir genes in these cells. 3) To explore the mechanisms by which macrophages take up, "process" and present antigen to lymphoid cells.

Methods: 1) Macrophages are purified from spleen and peritoneal exudate by adherence to glass and removal or inactivation of other cell types by various means including treatment with anti θ and C' and irradiation. 2) Macrophages are removed from lymphoid cell populations by passage over Sephadex G10. 3) Ia antigens and other surface markers are detected by immunofluorescence. Ia bearing cells are removed by treatment with alloantisera and C'. The synthesis of Ia antigens is measured by internal labeling with ^{3}H-leucine, followed by solubilization, immunoprecipitation and gel electrophoresis. 4) The immunologic function of macrophages is assessed by T cell activation as measured by thymidine incorporation in vitro, or by antibody formation by B cells in the in vitro induction of primary and secondary immune responses as measured by plaque formation.

Major Findings: When macrophages were examined for Ia antigens by indirect immunofluorescence it was found that spleen macrophages expressed more I region antigens than macrophages from the peritoneal cavity. This may reflect differences in their stages of differentiation, as spleen macrophages maintained under stimulating conditons in vitro demonstrated decreased amounts of detectable Ia. In addition, antigens determined by different I subregions were not equivalently expressed on normal spleen macrophages: the I-A subregions were detected on 57% of these cells as compared to 11% for the other subregions.

The significance of macrophage Ia has been explored using various populations of macrophages as accessory cells in in vitro immune responses. In the T-independent, macrophage-dependent B cell primary antibody response to TNP-Ficoll it was found that peritoneal (PE) macrophages functioned as well or better than an equivalent number of spleen macrophages, indicating that Ia antigens are not relevant in this cell interaction. Conversely, in the secondary T cell proliferative response to several antigens, Ag pulsed spleen macrophages functioned almost 50 fold better than PE macrophages, a functional enrichment directly paralleling the enrichment of Ia bearing macrophages.

Significance to Biomedical Research and the Program of the Institute: As macrophages function both in the induction of T and B cell immunity and can also function as cytotoxic cells in tumor immunity, an understanding of the mechanisms underlying the role of macrophages in the immune response may lead to new modes of therapy for cancer and other human diseases.

Proposed Course of Project: The function of macrophages in both T and B cell activation will be further explored using conventional and Ir controlled antigens in in vitro proliferative and plaque forming cell responses. The

role of Ia on these cells will be assessed by killing those macrophages bearing Ia antigens using subregion specific antisera and C'. The mechanism of Ag uptake and presentation by macrophages will be examined by internal and surface labeling of Ia in conjunction with uptake of radiolabelled antigens under Ir control, and by the use of various protein synthesis inhibitors during Ag pulsing. Allogeneic restriction of cell interactions between macrophages and T or B cells will be evaluated in both primary and secondary responses in vitro. Activation of T cell suppression will be attempted using Ag pulsed Ia^+ and Ia^- macrophages.

Publications

Cowing, C. and Dickler, H. B.: Expression of Ia antigens on murine macrophages: In Proceedings of the Third Ir Gene Workshop, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05066-01 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Antibody Production to TNP-Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	R. B. Levy	Postdoctoral Fellow	I NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Immunology Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mouse spleen cells sensitized in vitro to TNP-modified syngeneic spleen cells were tested for the generation of antibody-forming cells (as well as for cytotoxic function) by assaying the cultured lymphocytes in a plaque-forming cell (PFC) assay using TNP-modified sheep erythrocytes (TNP-SRC) as targets. A PFC response specific for the TNP-haptenic moiety was detected when the cultured spleen cells were from C57BL/10, B10.D2, or B10.A(5R) donors, whereas no response was detected in cultured lymphocytes from B10.A or B10.A(4R) donors. In contrast, spleen cells from all of the above mouse strains generated strong cytotoxic responses against TNP-modified self cells. These results indicate that the antibody response against the TNP hapten coupled to syngeneic cells is controlled by genetic factors mapping within the H-2 complex (possibly immune response genes) which do not affect T-cell-mediated cytotoxicity against TNP-self structures, and suggests that these antibodies and T-cell receptors recognize different antigenic specificities.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this project was to investigate whether mouse lymphocytes can be sensitized in vitro against TNP-modified syngeneic cells to generate TNP-specific antibody-producing PFC. If PFC could be generated, it was the purpose of this research to compare in different mouse strains the PFC and T-cell mediated cytotoxic responses against TNP-self and to determine what self components are recognized as carrier for PFC generation.

Methods Employed: Mouse spleen cells from different inbred mouse strains were sensitized in culture against TNP-modified syngeneic cells. The cells harvested from the cultures were assayed for PFC on TNP-modified SRC, and for cytotoxic activity on ^{51}Cr-labelled TNP-modified syngeneic lymphoid target cells.

Major Findings: TNP-specific PFC were generated by spleen cells from C57BL/10 and B10.A(5R) donors, but not from B10.A and B10.A(4R) donors. Cytotoxic responses against TNP-self were generated in the same cultures from all of the above strains. These results indicate that genetic factors mapping in the K and/or I-A regions of the H-2 complex control TNP-specific antibody-forming cell production.

Significance to Biomedical Research and the Program of the Institute: The finding that hapten-specific antibody can be produced when hapten is coupled to self carrier may have significance in autoimmunity. The observation that the ability to produce hapten-specific antibody on self carrier is controlled by H-2 raises the possibility that autoimmune phenomena may be linked to the MHC. This model also permits an analysis of the mechanisms leading to the production of cytotoxic effector cells and antibody-forming cells in the same cultures.

Proposed Course of Project: Studies will be performed to: (a) map the genes regulating TNP-PFC production and to understand their regulatory functions; (b) determine whether the response is T-cell dependent, and if so, to determine what self component(s) constitute the carrier moiety of the immunogen; (c) to determine whether this response can be obtained in vivo; and (d) to analyze the cellular events leading to PFC and cytotoxic effector cell production in the same cultures and/or animals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05067-02 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to TNP-Modified Syngeneic Lymphocytes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	J. S. Shaw	Clinical Associate	I NCI
	A.-M. Schmitt-Verhulst	Expert	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Primary cultures of peripheral blood leukocytes from a series of unrelated human donors were stimulated in vitro against TNP-modified autologous leukocytes. Weak primary proliferative responses were detected using ^{3}H-thymidine pulsing of the responding cells. The cultures were restimulated with TNP-modified or unmodified autologous or allogeneic leukocytes. Strong proliferative responses were detected preferentially in cultures restimulated with TNP-modified autologous cells, or in modified allogeneic cells sharing an HL-A A locus specificity with the modified primary stimulating cells.

PHS-6040
(Rev. 10-76)

Project No. Z01-CB-05067-02-I

Project Description

Objectives: The purpose of this project was to determine whether human lymphocytes can recognize and respond to TNP-modified autologous leukocytes in ways similar to that observed using mouse lymphocytes, and if human modified self reactivity can be detected, to determine if self HL-A coded products are involved in the recognition and to map such products within the HL-A complex.

Methods Employed: Human peripheral blood leukocytes were obtained from normal adult volunteers, separated on a Ficoll-Hypaque gradient, and sensitized in vitro in primary and secondary cultures against TNP-modified and unmodified autologous and allogeneic (unrelated) leukocytes. Proliferation of the responding lymphocytes was assessed by pulse-labelling with ^{3}H-thymidine at various times after primary and secondary culture.

Major Findings: Human peripheral blood leukocytes responded weakly to TNP-modified autologous cells in a primary culture. Following restimulation, the lymphocytes were found to give strong preferential responses against TNP-modified autologous cells or modified HL-A-matched allogeneic secondary stimulators when compared with modified unrelated, non-HL-A-matched secondary stimulating cells.

Significance to Biomedical Research and the Program of the Institute: This project is of fundamental immunological importance in that it provides the first known example of cell-mediated immunity involving self-recognition by human lymphocytes, and it can be used to test a number of major histocompatibility-linked immune phenomena in man which are known to occur in mice. It may also be useful for HL-A typing of human lymphocytes for possible new immune functions and for investigating immune response genes in man without having to immunize human subjects.

Proposed Course of Project: Studies will be carried out using lymphocytes from unrelated HL-A matched and unmatched individuals, and between members of families including those known to be recombinants within the HL-A complex in order to determine whether the proliferative response to TNP-modified self is HL-A linked. The response potential of lymphocytes from individuals will be compared for possible differences that could be controlled by immune response genes and linkage for subregions of HL-A will be tested. The TNP-modified self system will be studied for cytotoxic function.

Publications

Shearer, G. M., Schmitt-Verhulst, A.-M., Shaw, S., Pettinelli, C., Henkart, P., and Rehn, T. G.: The role of MHC genes in T-cell mediated responses to syngeneic modified cells. Proceedings of Symposium on Molecular and Cellular Biology, Park City, Utah. March 21-25, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05068-01 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunology of Model Membrane Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. A. Henkart	Senior Investigator	I NCI
OTHER:	C. K. Neels	Microbiologist	I NCI
	G. M. Shearer	Senior Investigator	I NCI
	A.-M. Schmitt-Verhulst	Expert	I NCI

COOPERATING UNITS (if any)

D. Wolf, J. Schlessinger, W. Webb, Department of Applied Physics;
E. Elson, Chemistry Department; Cornell University, Ithaca, NY

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.2	0.8

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amphipathic macromolecules have been synthesized as models for membrane proteins. Fatty acids were covalently linked to dextran derivatives of various molecular weights, and various amounts of hapten (TNP) and fluorophore (rhodamine or fluorescein) were also linked to the molecule. These molecules adsorb to all artificial and cell membranes tested. On planar lipid bilayers their lateral diffusion has been measured and patching induced upon addition of anti-TNP. On lymphocytes, these molecules patch and cap rapidly if exposed to anti-TNP; such capping is blocked by metabolic inhibitors. When adsorbed to the surface of lymphoid cells, these TNP molecules do not elicit the production of killer T lymphocytes nor are TNP-dextran target cells recognized or destroyed by killer T cells which do kill "TNP-self" target cells, i.e., cells whose surface antigens are modified by the reagent TNBS.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To design and synthesize well characterized amphipathic macromolecules which will insert into membranes. To measure the lateral diffusion of these molecules when inserted into artificial lipid bilayer membrane and a variety of cell membrane. To observe the effects of cross-linking the membrane-bound macromolecules. To see if killer T lymphocytes are capable of recognizing such foreign cell-surface molecules.

Methods Employed: The synthesis of the dextran derivatives containing TNP, rhodamine and stearic acid (TRSD) was accomplished by utilizing a combination of published reactions. The resulting water-soluble preparations were exposed at .01-3 mg/ml to membranes, and the non-adsorbed species washed away. Horizontal planar lipid bilayers were formed by the Mueller-Rudin technique. Lateral diffusion was measured by fluorescence photoblasting recovery. For cell bound TNP dextran derivatives, detection in the fluorescence microscope was by rhodamine-anti TNP.

Major Findings: The stearic acid dextran derivatives adsorb to all membranes tested. On lipid bilayers fluorescence showed that membrane concentrations of 10^2-10^4 molecules result from sensitization with .10-40 µg/ml of the rhodamine labelled derivative. Lateral diffusion constants of $0.3\text{-}4.5\times10^{-8}$ mm^2/sec are observed depending on the 2 dimensional concentration and bilayer lipids. Addition of anti-TNP (5 µg/ml) to membrane-bound TRSD at 10^2 molecules/μ^2 gives rise to patching, while addition of anti TNP to 10^4 TRSD molecules/μ^2 decreases the lateral diffusion constant to zero with no patching. Lateral diffusion of a lipid probe in the membrane is not affected by the presence of TRSD or its subsequent crosslinking. The dextran derivative substituted with TNP and stearic acid (TSD) binds to lymphocytes as measured by immunofluorescence using rhodamine anti-TNP. If allowed to incubate at 37°C, over half of the mouse spleen lymphocytes rapidly form caps. This capping process is inhibited by blockers of metabolic energy production, cytochalasins B and D, and local anesthetics. Other cells such as fibroblasts and tumor cells also form caps when surface bound TSD is crosslinked by anti-TNP. Cell surface TSD does not elicit the differentiation of killer T lymphocytes under in vitro conditions where allogeneic and TNBS modified cells do so. Killer T cells which are specific for "TNBS modified self" do not kill or recognize TSD modified cells, even though there is a quantitatively equivalent number of TNP groups and the H-2 antigens are recognizable by allogeneic effector cells.

Significance to Biomedical Research and the Program of the Institute: We have devised a model system for studying membrane bound antigens in a controlled system. Many of the properties of these molecules mimic the properties of natural cell surface antigens--patching, capping and lateral diffusion. Since tumor antigens are natural cell surface antigens and since there is other evidence that malignant transformation alters cell surface components, it is important to understand the behavior of such systems. We believe the model system we have devised is of real importance towards this end.

Proposed Course of Project: We are currently extending the studies on planar lipid bilayers to obtain more quantitative data on the number of bound antibody molecules and the degree of patching of various TRSD membrane densities. Data on lateral diffusion of TRSD bound to cells will be obtained. We are also attempting to modify various proteins with lipid substituants in an attempt to use them instead of polysaccharides, especially as cell surface bound immunogens.

Publications

Wolf, D. E., Schlessinger, J., Elson, E. L., Webb, W. W., Blumenthal, R., and Henkart, P.: Diffusion and patching of macromolecules on planar lipid bilayer membranes. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05069-01 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Expression of Ia Antigens on Functional Cell Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Ahmann	Clinical Associate	I NCI
OTHER:	R. J. Hodes	Head, Immunotherapy Section	I NCI
	D. H. Sachs	Head, Transplantation Biology Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been demonstrated that pre-treatment of mouse lymphoid cells with anti-Ia (I-region-associated) sera and complement abrogates the T cell proliferative response to Con A and lentil lectin, but not PHA. Genetic studies have shown that the Ia determinants expressed on Con A reactive cells are coded for by at least two different subregions of the I-locus. Anti-Ia sera and complement have also been shown to prevent the generation of CML (cell-mediated lympholysis) suppressor cells induced by 5-day culturing of normal spleen cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Ia antigens have been associated with many immunologically-related phenomena. Using defined Ia alloantisera, it has been possible to map the genes to subregions that encode particular Ia determinants. The objective of this study is to use these antisera to identify markers for cells involved in specific immunological function (e.g. Con A reactive cells, suppressor cells, helper cells, etc.) and from this hopefully to clarify the mechanisms of action of these cells, as well as the role of Ia antigens in these responses.

Methods Employed: Cells are treated with anti-Ia sera and complement and the resulting Ia-resistant cells are cultured in vitro with mitogens, and the proliferative response is determined. Cell separation techniques are being employed to determine which cell is eliminated by the anti-Ia sera and complement treatment. Ia resistant cells are also treated for their ability to generate CML and respond in an MLR (mixed lymphocyte reaction) in culture. Absorption of the anti-Ia sera are performed to obtain more restrictive activity against Ia determinants encoded by particular I subregions.

Major Findings: It was shown that treatment of murine lymphoid cells with anti-Ia sera and complement significantly reduced the response of these cells to Con A at all doses tested. The response to PHA was not affected but the LPS response was completely abrogated. Using cells from congenic intra-H-2 recombinant strains for absorption of the antisera, it was shown that this activity was confined to the I-A subregion. Furthermore testing for anti-Ia reactivity toward determinants whose gene maps between I-B through S also revealed the ability of this antisera to decrease Con A reactivity.

Treatment of lymphoid cells with anti-Ia sera and complement did not prevent them from responding in CML or MLR. However, such treatment abolished the generation of CML suppressor cells by 5-day culturing of normal spleen cells. It is concluded that a cell population required for Con A response expresses Ia antigens; the in vitro generation of at least one suppressor cell activity also requires participation of an Ia-positive cell population. In contrast, no such requirement for Ia positive subpopulations has been detected for responses to PHA, MLR responses, or the generation of cytotoxic effector cells.

Significance to Biomedical Research and the Program of the Institute: Ir gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products, Ia antigens, in CML, MLR, and T cell-mediated suppression should provide insight into mechanisms of control of the immune response. Such insight would be relevant to evaluation of the host response to allograft and tumor challenge.

Proposed Course of Project: Studies are in progress to complete the genetic mapping of the genes controlling the Ia determinants in the Con A proliferative response as well as to determine on which cell they are expressed. Genetic studies will also be performed to determine which gene(s) control expression of the Ia determinants necessary for the generation of a suppressor cell in CML. Work will also be done on the role of Ia antigens in an in vitro antibody synthesis techniques.

Publications

Ahmann, G. B., Sachs, D. H., and Hodes, R. J.: Genetic analysis of Ia determinants expressed on Con A reactive cells. In Proceedings of the Symposium on Regulation of the Immune System: Genes and the Cells in Which They Function (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05070-01 I

PERIOD COVERED

July 1, 1976 to May 1, 1977

TITLE OF PROJECT (80 characters or less)

The Effect of Tumor Cells in Regulating Cell Mediated Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Head, Immunotherapy Section	I NCI
OTHER:	H. Koren	Department of Immunology, Duke University	

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been demonstrated that the ability of lymphoid populations to generate cell mediated cytotoxicity against alloantigens in vitro can be completely abrogated by the depletion of adherent accessory cells. Activity is totally restored in these functionally depleted populations by the addition of small numbers of radioresistant theta negative peritoneal cells (macrophages). In addition it has been demonstrated that this accessory cell function can also be reconstituted by tissue culture maintained syngeneic tumor cells. These findings are interpreted as demonstrating that cells other than adherent macrophage like cells are capable of providing accessory cell function for cell mediated immune responses in vitro.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The generation of cell mediated cytotoxicity effective against allogeneic or syngeneic target cells, including tumor cells, has been characterized as a T cell function. These investigations were undertaken to characterize the role of other, accessory, cells in the generation of these responses.

Methods Employed: The general methodology employed in generating and assaying cell mediated cytotoxicity in vitro has been previously described in detail. In these experiments G-10 Sephadex passage was used as a method for depletion of adherent macrophage-like cells from responding lymphoid populations. In order to achieve adequate depletion of macrophages, this method was combined with exposure of lymphoid cells to iron particles followed by magnet separation and Ficoll-hypaque gradient centrifugation. In order to provide a source of sensitizing or stimulating cells for in vitro sensitization which was itself devoid of macrophage activity, tissue culture lines of allogeneic tumor cells were used throughout as a source of alloantigenic cells. Macrophage rich populations were obtained by washout of peritoneal cells followed by irradiation and anti-theta plus complement treatment of these cells.

Major Findings: It was observed that treatment of spleen cells with G-10 Sephadex passage followed by iron-magnet separation resulted in a population which was unable to generate significant cytotoxicity to allogeneic tumor cells in vitro. This activity could be reconstituted by the addition of 2-mercaptoethanol or enriched peritoneal macrophages. In addition, relatively small numbers of syngeneic tumor cells, $P388D_1$ (a macrophage like tumor line), P815, or P388 (nonmacrophage like lines) were also able to fully reconstitute the responsiveness of macrophage depleted populations.

Significance to Biomedical Research and the Program of the Institute: T cell dependent cell mediated cytotoxicity has been considered to be of potential relevance to host resistance to tumor. The findings described above are relevant in the information which they provide with respect to requirement for accessory cells in generation of this immune response in vitro. In addition the fact that certain tumor cells, at least in vitro, are capable of providing an effective accessory cell function suggests an additional dimension of the complex interaction between host and tumor.

Proposed Course of Project: The investigations reported above have essentially concluded the immediate objectives of this project.

Publications

Koren, H. S., and Hodes, R. J.: Accessory cell functions of mouse tumor cells in the generation of cytotoxic T lymphocytes in vitro: Replacement of adherent phagocytic cells by tumor cells or 2-ME. Eur. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05071-01 I

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Affect of Sendai Viral Infection on the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. M. Nadler	Clinical Associate	I NCI
Other:	R. J. Hodes	Head, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

M. Whiteman, Microbiological Associates, Bethesda, Md.
M. J. Collins, Director of Animal Diagnostics, Microbiological Associates

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To investigate the effect on the immune response of Sendai viral infection, several classical in vitro immunologic parameters were studied. Detailed time courses and dose responses of Sendai viral infection reveal no suppression of the immune response as measured by responses of spleen cells from infected mice to mitogens, proliferative mixed lymphocyte reaction, and cell-mediated lympholysis. Studies done in syngeneic normal mouse serum or sera from Sendai infected animals showed a moderate enhancement of the in vitro immune responses to mitogens and alloantigens by serum from infected animals.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Study of the immune response of animals infected with Sendai virus was undertaken to investigate whether this epizootic lethal murine virus caused immunosuppression as measured in in vitro immunologic systems. Further, if immunosuppression was demonstrated, analysis of the mechanism of suppression by a natural viral infection would be undertaken.

Methods Employed: BALB/c or 129/J mice were intranasally infected with varying dosages of Sendai virus. At varying times after innoculation mice were sacrificed and spleens were explanted. Single-cell spleen cell suspensions were prepared and placed in in vitro culture with either mitogens (PHA, Con A, or LPS), allogeneic cells, or sheep red blood cells. Assays included: 1) assay of cell proliferation in response to mitogens or allogeneic cells (MLR) measured by thymidine incorporation; 2) Chromium 51 release assays of cell-mediated cytotoxicity after in vitro sensitization to alloantigen (CML); 3) in vitro antibody responses to sheep red blood cells; and 4) antibody production in vivo to Sendai virus assayed by complement fixation or inhibition hemagglutination technique.

Major Findings: After infection of mice with Sendai virus, there was no detectable suppression of immune response by spleen cells as assayed by mitogen responses, MLR, CML, and antibody production. Comparison of a mouse strain which is highly susceptible to Sendai infection (129/J), and a more resistant strain (BALB/c), revealed no difference in the immune response as measured by in vitro assays. When sera from syngeneic animals, either normal or infected with Sendai virus, were tested in culture, it was found that the in vitro proliferative responses to mitogens or in MLR were enhanced by sera from infected mice. There was thus no evidence for suppression of immune responses in vitro or in vivo following infection with epizootic Sendai virus.

Significance to Biomedical Research and the Program of the Institute: The effect of viral infection on the immune response is of potential significance in terms of host resistance to infectious challenge and tumor growth. The present work reveals lack of generalized immunosuppression by Sendai virus as measured by in vitro immunologic correlates. It does not rule out more specific forms of immunosuppression or suppression not detectable by the assay systems employed. The enhancement of mitogen and MLR responses produced by serum from virus-infected mice is of uncertain relevance to the in vivo immune competence of these mice.

Proposed Course of Project: The studies reported have completed the major objectives of this task. Further analysis will be undertaken of the mechanism of enhanced MLR and mitogen responses produced by serum from Sendai infected animals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05072-01 I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Affect of Viral Infection on the Serum Amyloid Precursor Protein (SAA)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	L. M. Nadler	Clinical Associate	I NCI
Other:	K. P. W. J. McAdam	Visiting Associate	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION
Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been demonstrated that Sendai viral infection produces a rise in the serum amyloid precursor protein (SAA). The time of the peak SAA response is later (9 to 11 days) than the peak of virus production (4 to 5 days). The rise in SAA parallels the development of pneumonia and therefore suggests that inflammation may be required for the production of SAA. An analysis of the course of induction of interferon and that for SAA has been carried out after viral challenge in an attempt to determine whether SAA production is correlated with viremia per se, or with the inflammatory process which is secondary to viremia.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objective of this work was to define the response of serum amyloid precursor protein (SAA) to viral infection. Further, this response would be correlated with immunologic and viral parameters in order to study the physiologic stimuli which induce SAA production.

Methods Employed: Standard techniques of intranasal viral innoculation of Sendai virus were employed. BALB/c or DBA/2 mice were used throughout. Animals were bled after receiving varying doses of virus and the sera were tested by solid phase radioimmunoassay for SAA. The time course of SAA production was compared to changes in animal weight, lung weight, virus titre in the lungs, interferon titre, and lung pathology.

Major Findings: Sendai viral infection leads to production of SAA. The peak of SAA titre in response to this stimulus occurs significantly later than the responses to other inducing stimuli which have been studied (9 to 11 days vs 1 to 2 days). The rise in the SAA titre correlates well with the development of pathologic pneumonia as measured by gross pathology, lung weight, and animal weight loss. The kinetics of the rise in SAA titre differ significantly from the time course of viral replication in the lung. SAA reaches a peak 4 to 6 days later than the peak of viral titre, suggesting that the inflammatory response of the viral pneumomitis, rather than virus itself, may be responsible for the SAA rise. Work presently in progress suggests that intravenous virus fails to stimulate SAA production, a finding which may permit further analysis of the relationship between SAA production and viral challenge. Correlation of intravenous viral induction of interferon with SAA production will hopefully permit dissociation of these two acute phase reactants.

Significance to Biomedical Research and the Program of the Institute: The present study defines the relationship of circulating SAA to viral infection. The observation that the SAA response occurs after the development of pneumonia and not at the time of maximal viral replication or viremia suggests that SAA may be mediated by or may be a product of the inflammatory response. This relationship may permit further elucidation of the physiology of the SAA protein and permit investigation of the pathways (i.e., mediators) which stimulate SAA production. SAA has been found to be elevated in patients with a variety of cancers. In addition, recent in vitro work would suggest that SAA may be immunosuppressive. Therefore, further study may lead to insight into the relation of the SAA protein and the immunosuppression associated with cancer in man.

Proposed Course of Project: Study of the relation of SAA to the mediators of inflammation and the relation of SAA to immunoregulation are future areas of interest.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05073-01 IP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modification of Glycoproteins in Membranes of Animal Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shifrin	Chemist	IP DCBD NCI
Other:	C. Parrott	Chemist	IP DCBD NCI

COOPERATING UNITS (if any)

J. C. Lee, Johns Hopkins University

LAB/BRANCH
Office of the Associate Director, Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20014

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project was to learn the techniques of culturing animal cells and to develop methods of chemically modifying and labeling the glycoproteins of cell membranes with fluorescent dyes. The topics of special interest include: 1) Treatment of tissue culture cells with specific glycohydrolases and examining the effect of these enzymes on cell growth; 2) Specific chemical modification of sugar moieties of cell surface glycoproteins followed by reaction with a fluorescent dye.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this project is to determine the role of the sugar moieties of cell surface glycoproteins in macromolecular interactions, in cell-cell interactions and in cell growth. Tissue culture cells were treated with neuraminidase from Clostridium perfringens and were examined by phase contrast microscopy. The asialo-glycoproteins thus produced were then treated with galactose oxidase followed by reaction with various fluorescent amines.

Methods Employed: Visible and ultraviolet absorption spectroscopy, fluorescence spectroscopy, sedimentation velocity ultracentrifugation, SDS-polyacrylamide gel electrophoresis, radioactive tracer techniques, tissue culturing of animal cells, chemical and enzymatic modification of glycoproteins, isolation of cell membranes and microscopic examination of cells by phase contrast and fluorescence microscopy.

Major Findings:

A. As an introduction into the study of chemical modification of specific sugar moieties, with the collaboration of Dr. Y. C. Lee at Johns Hopkins University, we introduced galactose residues into L-asparaginase from Escherichia coli, a protein with which we have had extensive experience and whose physicochemical properties are well understood. The substitution of 5% or 50% of the lysyl residues with a galactose moiety does not destroy enzyme activity nor does it cause the tetramer to spontaneously dissociate. Further studies on the influence of the galactose residue on subunit interactions were not continued because our supervisor was not interested in this line of investigation.

B. T AL/N and SV AL/N cells were grown to 60% confluence in MEM containing 10% fetal calf serum. The serum was removed by several washes with phosphate buffered saline and the cells were covered with Eagle's minimal medium without serum. The medium was removed and the cells were incubated with neuraminidase for several hours at 37°C. The incubation medium was collected and the sialic acid content determined by a sensitive fluorometric thiobarbituric acid method. The asialo-cells were placed into complete medium again and grown overnight. These treated cells grew to the same extent as the control suggesting that a terminal sialic acid residue on the glycoproteins is not necessary for normal growth in tissue culture.

Our results indicated that the sialic acid content of SV40 transformed cells is slightly higher than the non-transformed cells which is contrary to that found in the literature. These studies are currently being repeated.

The asialo-cells were again washed free of serum and were treated with galactose oxidase overnight at 37°. This treatment converts the primary alcohol group to an aldehyde which was then reacted with ethylenediamine-naphthylsulfonic acid (EDNA), a highly fluorescent amine. The cells were thoroughly washed to remove unreacted amine and were examined under the

fluorescence microscope. The surface of the cells were highly fluorescent.

Studies are being initiated into an examination of γ-glutamyltranscarboxylase in normal and transformed cells.

Significance to Biomedical Research and the Program of the National Cancer Institute:

Specifically labeling the cell surface of tissue culture cells will allow us to follow the movement of the cells in culture and to examine the effect of SV40 transformation on cell-cell recognition. An understanding of the role of the cell surface in cellular interactions may help elucidate the difference between normal and transformed cells.

Proposed Course of the Project:

The influence of various sugar hydrolases on cell surface glycoproteins as a means of modifying the architecture of the cell surface and the cell shape is being continued. Chemical modifications of the polypeptide as well as the glycopeptide portions of the cell surface are also being planned.

Publications:

Parrott, Catherine L. and Shifrin, Sidney: The effect of trinitrophenylation on subunit interactions in L-asparaginase. Biochim. Biophys. Acta 445: 437-445, 1976.

Parrott, Catherine L. and Shifrin, Sidney: A spectrophotometric study of the reaction of borohydride with trinitrophenyl derivatives of amino acids and proteins. Biochim. Biophys. Acta 491: 114-120, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05074-I

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Differential Susceptibility to Sendai Viral Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Nadler	Clinical Associate	I NCI
OTHER:	R.J. Hodes	Senior Investigator	I NCI

COOPERATING UNITS (if any)
Microbiological Associates, Bethesda, Maryland

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Infection of animals via natural contagion demonstrates that animals are more susceptible to Sendai virus derived from syngeneic animals than to virus derived from allogeneic animals. Priming with allogeneic spleen cells improves survival of animals which are challenged with virus derived from that allogeneic strain. Experiments employing direct intranasal viral challenge confirm these findings. These data suggest that susceptibility to Sendai virus may be influenced by recognition of host derived histocompatibility antigens on the viron.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: This project was undertaken to investigate whether there is a differential susceptibility to Sendai viral infection when the infecting virus originates in animals syngeneic or allogeneic to the recipient strain. The advantage for animals to be able to identify virus from an allogeneic animal would allow rapid recognition of the virus as foreign and lead to abortion of the infection. If this recognition process occurs as the literature would suggest via an incorporation of host cell surface (probably histocompatibility antigen) into the viral envelope then this protection from infection may have also led to one of the evolutionary pressures leading to the polymorphism of the histocompatibility antigens.

Methods Employed: Mice of histocompatibility $H2^b$ (129/J) or $H2^d$ (DBA/2) were used in most experiments. Sendai virus derived from DBA/2 or 129/J lung homogenates was employed for viral infection. Animals were infected with Sendai virus either by intranasal route or by natural contagion from infected mice. Susceptibility to viral infection (measured by survival) was tested by infection of mice with virus derived from syngeneic or allogeneic animals. $H2^b$ or $H2^d$ mice were primed with spleen cells from mice of $H2^b$, $H2^d$, or $H2^s$ histocompatibility. These primed mice were infected with virus derived from $H2^b$ or $H2^d$ mice. Susceptibility was measured by survival of infected animals.

Major Findings: In natural contagion experiments, animals of the $H2^b$ haplotype had a longer survival than animals with the $H2^d$ haplotype if they were infected with virus derived from animals of the $H2^d$ haplotype. This was true in the converse and thus mice challenged with virus derived from syngeneic animals have a much shorter survival than animals infected with virus derived from an allogeneic source. In experiments where titrated amounts of virus were introduced intranasally, prolonged survival occurred only in animals challenged with virus derived from the allogeneic source. DBA/2 mice primed with $H2^b$ or $H2^s$ spleen cells were challenged with 129/J ($H\text{-}2^b$)-derived Sendai virus. Survival was significantly enhanced only in those mice which had previously been primed to $H2^b$. A viral equivalent of 100 LD_{50} produced a survival of 66% and a viral equivalent of 10 LD_{50} produced a survival of 89% in only $H2^b$ primed recipients receiving $H2^b$ virus. Irrelevant immunization with spleen cells of $H2^s$ did not improve survival.

Significance to Biomedical Research and the Program of the Institute: The prolongation of survival by Sendai viral infection from an allogeneic viral source would suggest a recognition of the virus as foreign by the recipient animal. The explanation for this protection is best explained by the hypothesis that virus incorporates a portion of the host cell surface into the viral coat. This foreign antigen borne on the viral coat would allow animals that could recognize this antigen as foreign to rapidly respond to the virus. The recognition of viral particles via histocompatibility antigen on the viral coat would make it advantagous for the animal to have a widely differing set of histocompatibility antigens. This point may address one of the proposed theories for the polymorphism of the histocompatibility antigen.

Proposed Course of Project: At this point we have no proof that the protection is conferred via histocompatibility antigens. Report of protection via immunization in congeneic mice, differing only at the major histocompatibility locus would permit this proof. The demonstration that histocompatibility antigen was on the surface of the virus by neutralization experiments is in order. Finally, demonstration that an unimmunized host infected with allogeneic virus develops a measurable immune response to the alloantigen would support this theory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05526-09 IP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Spontaneously or by SV40 Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Head, Macromolecular Biology Section	IP DCBD NCI
Other:	S. W. Luborsky	Chemist	IP DCBD NCI
	C. Chang	Visiting Fellow	IP DCBD NCI
	J. Küster	Visiting Fellow	IP DCBD NCI
	D. Winterbourne	Visiting Fellow	IP DCBD NCI

COOPERATING UNITS (if any)

G. Khoury, LDNATV, DCCP, NCI
R. G. Martin, LMB, NIAMDD
R. Weil, University of Geneva, Switzerland
D. M. Livingston, Sidney Farber Cancer Center, Boston

LAB/BRANCH

Office of the Associate Director, Immunology Program

SECTION

Macromolecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell surface membrane, how it may change in transformation to malignancy, either spontaneously, or as induced by SV40 virus. Currently research is carried out on 1) Changes in glyco-conjugates, including glycoproteins and mucopolysaccharides, and correlation with cell growth properties in tissue culture and in vivo in the syngeneic and in the nude mice; 2) Controls of expression and methods of purification of SV40 specific T antigens, surface antigens and tumor specific transplantation antigens; 3) Selection of phenotypic and genotypic revertant tumor cells which lose SV40.

Project Description:

Objectives: To elucidate certain biochemical and biological changes on the cell surface which occur during transformation to malignancy in mouse cells.

Methods Employed: Labelling glycoproteins, mucopolysaccharides, proteins and glycolipids, also of nucleic acids with radioactive precursors during cell growth in culture. Novel fractionation techniques for isolation of (labelled) subcellular components, including cell surface membranes. Isolation and characterization procedures in thin-layer and column chromatography, gel electrophoresis, and in autoradiography of gel slabs. Analytical and preparative ultracentrifuge studies. Enzymology techniques pertinent to intermediary metabolism of carbohydrates. Serum mediated cytotoxicity assays as measured by release of radioactivity from lysed cells. Cell growth dynamics in culture, and also in syngeneic and in nude mice.

Major Findings:

I. Changes in cell membrane components in exponentially growing mouse fibroblast cells.

Clonal derivatives of mouse embryo fibroblast cell lines were developed, with controlled genealogy and careful matching of sister clones. Sister clones were obtained with differing tumorigenicity (sarcoma) in the syngeneic AL/N or Balb/c mice, including highly tumorigenic cells ($TD_{50}=10^2$ cells/mouse). These cells appeared spontaneously in tissue culture, with no obvious or suspected cause for their malignant transformation (cf. no detectable "C" type viruses). The normal (non-tumorigenic) and the spontaneously transformed tumorigenic clonal cell lines were then infected with SV40 virus, and both SV40 transformed (T antigen positive) and non-transformed reclones were obtained. The various cloned cell lines were then characterized with regard to their growth in the syngeneic and in the nude mice (TD_{50}), and also to their growth properties in tissue culture both in attachment to substratum and in viscous suspension medium independent of anchorage. These biological parameters were then correlated with various biochemical measurements of the cell surface membrane.

A. Protein changes. To externally label the cell surface, a new sensitive and non-distructive method was developed, based on the reaction of pyridoxal phosphate which forms a Schiff base preferentially with the lysine ε-amino groups of proteins and glycoproteins, followed by reduction with KB^3H_4 (original work with S. Pancake, now at Georgetown University). Internal metabolic labelling of proteins was by radioactive amino-acids. All cells were labelled in their optimal, exponential phase of growth. When preparations from these cells enriched in cell surface membrane were analyzed in protein constitution, one (glyco)protein component (apparent molecular weight about 250,000, similar to the so-called LETS of glycoprotein) was observed to be reduced or absent consistently in an SV40 virus transformed cell line, when compared with the normal cell line. This is the first clear

observation on such difference when both type of cells are in their exponential growth phase. (The previous observations generally showed that the LETS glycoprotein in normal cells is especially high when the cells are arrested in their G_0 phase by density or by serum starvation, but it is much lower in the exponentially growing normal cells; in the virally transformed cells which keep growing in their post-confluent stage there is low amount of LETS glycoprotein.) During exponential growth in metabolic labeling experiments with ^{14}C-labelled mixed amino acids we also demonstrated a consistent decrease in the biosynthesis of the same 250,000 MW protein in the SV40 virus infected subclone, as compared to an uninfected sister subclone.

This deficiency in a single membrane protein component appears to be a common correlate of the presence and functioning of the SV40 genome, as expressed by the various "early" SV40 antigens, which have been detected, solubilized and partially characterized by appropriate biologic assays (see below). These antigens have a molecular weight at or below 100,000; obviously they represent a small, and by the above biochemical methods undetectable fraction of the proteins in the cell membrane.

No correlation was discernible in exponentially growing cells between membrane protein constituents, and the final saturation density. Apparently, in these cell lines, the ability of cells to grow to high density in monolayers in attachment is not a simple correlate of biochemically detectable protein change in the membranes.

The ability of all SV40 transformed cells, including the clonal cell lines, to grow without anchorage in viscous Methocel suspension as contrasted to the corresponding non-SV40 transformed cells including the T antigen negative sister subclones, appeared to correlate with the observed membrane difference in these cells. However, further work is necessary before it can be concluded that the reduced amount of the high molecular weight (glyco-)protein constituent(s) is a general correlate of anchorage dependent cell growth. Such work is in progress in other (Balb/c) matched clonal cell lines.

There was no simple correlation evident in the cell lines investigated between tumorigenicity and any membrane protein changes detectable by the methods employed. Both the normal cells, the highly tumorigenic clonal cell lines and its T antigen negative subclones, had similar, significant and well detectable amount of the 250,000 molecular weight protein component. It was shown that the SV40 transformed cells have lower tumorigenicity than the corresponding T antigen negative cells because the SV40 specific cell surface and transplantation antigen(s) cause cellular recognition and rejection in the syngeneic mouse (see below). Thus the observed difference in the 250,000 molecular weight cell membrane protein, is not a (direct) correlate of acquired cellular tumorigenicity in the genealogically related well matched cell lines. This is consistent with a recent study which showed that spontaneous neoplastic transformation in culture did not lead to the loss of the LETS protein, and transformation induced by chemical carcinogens or by viruses led to the loss of the LETS protein in some, but not in all cases (Hynes et al.). In another recent paper loss or different distribution of the LETS proteins on the surface

of cells was claimed to correlate with the oncogenic phenotype in certain other (adeno virus-2 transformed rat) cells; but not with the portion of the adeno virus-2 genome which is thought to be necessary for the maintenance of the transformed phenotype in culture (Chen et al., 1976, from Cold Spring Harbor). Obviously further work is necessary on genealogically related and well matched clonal cells which are normal or tumorigenic, which have or do not have the appropriate portions of the viral genes inserted. Such work is now possible and is being carried out with our clonal cell lines developed for this purpose (see below).

B. Analysis of various glycoconjugates. To carefully compare a pair of exponentially growing cell lines in their glycoconjugates, one type of cell was grown with ^{3}H, and the other with ^{14}C glucosamine, under identical conditions. Three culture compartments have been examined: 1) the medium, 2) material easily released from the cell surface without disrupting or altering the viability of the cells by gentle trypsin treatment, and 3) the trypsinized but still viable cells. These compartments from the different cells were mixed. Papain digestion of each compartment was used to eliminate excess proteins. The macromolecular (non-dialyzable) residues were separated by ion exchange. A computer program and printout was developed to give immediate comparisons of the two type of cells in the various separated glycoconjugate (also useful for any other pair of different radioactive labelled) components. Hyaluronic acid, heparan sulphate and chondroitin sulphate as well as a complex mixture of glycopeptides were found in the medium, the trypsinate and the trypsinized cells, although the distribution was not uniform. A spontaneously transformed parent and two clonal daughter cell lines had very similar in vitro growth properties, including their plating efficiency in viscous medium. However, the tumorigenicity of these cells, determined in syngeneic mice, was found to differ. All three cell lines were found to have rather similar glycoconjugate distributions, although a slight relative increase in labelled hyaluronic acid was found in the more tumorigenic mass cell line than either of the less tumorigenic daughter clones. This difference was most noticeable between those cells whose tumorigenicity differed by the largest amount. The finding implies that increased secretion of hyaluronic acid may benefit tumor growth. This is consistent with some other observations scattered throughout the literature, but not done previously with carefully matched cell pairs and by the quantitative and automatically normalizing comparative technique employed by us. The biochemical basis for the difference and its possible broader or general occurrence is under investigation in other matched cell lines (with D. Winterbourne).

Other work on modification of glycoproteins in cell surface membrane has begun, and is being reported separately (S. Shifrin, Project No. Z01 CB 05073-01 IP).

II. Controls of expression and methods of purification of T, surface, and tumor specific transplantation antigens.

The control of the expression of the early (A) region of a resident SV40 genome in an SV40 transformed cell attracts increasing attention. While on

the one hand it is responsible for the initiation and maintenance of the transformed cell growth phenotype (as far as tissue culture growth property), it apparently also encodes the nuclear T, and possibly the other SV40 specific antigens, such as the cell surface and the tumor specific transplantation antigens.

A. Detergent solubilization and partial purification of nuclear T, surface (TSSA), and transplantation (TSTA) antigens from SV40 transformed murine cells (with S. W. Luborsky and C. Chang) is reported in Project No. Z01 CB 05544-09 IP. The major results show that the three SV40 specific antigens T, TSSA and TSTA co-purify through several steps, that a reasonably purified T antigen preparation is highly active as TSTA, and that the TSTA activity (molecule) interacts with DNA and may have a gene regulatory role, similar to what was found by others for T antigen.

B. Study of the relationships of the T, TSSA and TSTA was carried out in a collaborative work (with C. Chang, R. Martin, NIAMDD, and D. M. Livingston, Sidney Farber Cancer Center, Boston). The study often employed temperature sensitive A mutant SV40 virus transformed cells. The results can be summarized as follows:

The simian virus 40-induced tumor-specific surface antigen(s) (TSSA) and tumor-specific transplantation antigen(s) (TSTA) were detected in cells transformed by wild-type or temperature-sensitive mutant simian virus 40, by an antibody-mediated cytolytic assay for TSSA, and an immunization test for TSTA. Cells transformed by most tsA mutants, which lose their transformed phenotype when grown at nonpermissive temperatures, nonetheless do express TSSA and TSTA as well as T-antigen at both temperatures.

A unique rat embryo cell line (A28-RE) transformed by a temperature-sensitive A mutant (isolated by Osborn and Weber) that loses T antigen during exposure to the nonpermissive temperature, was found also to lose TSTA. On the other hand, a more typical tsA-transformed cell line (isolated by Martin and Chou) expressed both T antigen and TSTA at the nonpermissive temperature. T antigen in lysates obtained from A239-MB cells was found to be three to four times more thermolabile by complement fixation than T antigen obtained from wild-type-transformed cells. In contrast to the T antigen which even in wild-type-transformed cells was completely destroyed in <10 min. at 50°C, the TSTA assayed *in vivo* by tumor rejection and the TSSA measured by an *in vitro* cytolytic assay, were thermostabile. Even after 24 hours of incubation of extracts at 50°C, high levels of TSTA and TSSA activity were present. Since these surface antigens when obtained from cells transformed by tsA mutants were also thermostabile, they could not be distinguished from the wild-type antigens. These results (a) indicate a coordinate expression of T antigen and TSTA in transformed cells; (b) confirm that T antigen is virus encoded; and (c) suggest that the antigenic and immunogenic determinants that characterize T antigen and TSTA activities are to some extent distinct.

The expression of SV40 TSTA in SV40-infected virus producing CV-1 monkey cells has been assayed, using detergent extracts of cell membranes and cytosol

to immunize mice prior to an SV40 ascites-tumor challenge. TSTA is expressed during infection of CV-1 cells by wild-type SV40 and by a temperature-sensitive A mutant. Following infection by the tsA mutant, TSTA (like the T antigen but unlike viral DNA, capsid proteins, and mature virus) appears both at restrictive (40.5°) as well as at permissive (33°) temperatures. Kinetic studies show that the initial appearance of TSTA and T antigen is concurrent.

The T antigen and the TSTA may be the same polypeptide, which, however, differs in the antigenic expression, for example, for reasons of difference in the secondary or tertiary structure. Three findings are pertinent:

a) Nuclear preparations (the usual source of T antigen) from human SV40 transformed cells contain 2 to 5 times higher levels of TSTA activity than cytoplasmic or cell membrane enriched preparations.

b) The solubilized fractions which were enriched in T antigen activity, were also enriched in TSTA activity (as reported in Project No. Z01 CB 05544-09 IP).

c) Purified T antigens from nuclei which were homogeneous by SDS PAGE, had a molecular weight of approximately 92,000, and were most highly active as TSTA.

The SV40 T and TSTA studies offer an opportunity to identify and fully define a strong and relatively easily characterizable antigen molecule which is responsible for the rejection of tumor cells.

C. Study of enhancing antigenicity of spontaneously transformed tumor cells after infection by SV40 (associative recognition) (with C. Chang).

Spontaneously transformed cells, unlike SV40 transformed cells, have very low antigenicity, if any. Attempts were carried out to study the humoral and cellular response against a spontaneously transformed cloned cell T AL/N C13, and its SV40 transformed derivative subclone (see below), with the hope that the weak antigenicity against the spontaneously transformed highly tumorigenic T AL/N C13 cell might be enhanced after further transformation by SV40. AL/N mice were immunized with T AL/N C13 or with its SV40 transformed subclone. Serum, peritoneal exudate and spleen and lymph node cells were obtained. The antibody response was measured with a highly sensitive assay based on interaction of the antibody molecule on the cell surface with I^{125} labelled bacterial "protein A". The cellular response was measured by Cr^{51} release with a cell mediated cytotoxicity assay. Significant killing of the SV40 transformed cells were found by immune peritoneal exudate cells, but not by the spleen or lymph node cells. The cell mediated cytotoxicity appeared to be specific to SV40 transformed cells. Against the spontaneously transformed cells no cellular cytotoxicity was detected using peritoneal exudate or spleen or lymph node cells from mice immunized with either the spontaneously transformed T AL/N C13 cells or by the SV40 transformed derivative subclone cell.

D. A study on the in vivo processing of T antigen and TSSA has also been initiated (with C. Chang, and collaborative work with the group of R. Weil, University of Geneva). The SV40 transformed murine cell lines, mouse, rat and hamster cell lines have been labelled with S^{35} methionine. Subcellular fractions such as nuclei, cytoplasma, microsomal and membrane fractions were obtained. Radioimmuno precipitations were then carried out with hamster antiserum and mouse SV AL/N (cytolytic) antiserum. Specific antigens were found after SDS PAGE and autoradiography, characteristic to SV40 transformed cells. Work is in progress to identify and define the antigens, with the prospect of understanding the different intracellular processing of the T antigen, the TSSA, and the TSTA.

III. Selection of phenotypic and genotypic revertant tumor cells which lose SV40. (With J. Küster, C. Chang and G. Khoury)

A spontaneously transformed highly tumorigenic cell line (T AL/N) was cloned, and the resulting clonal derivative T AL/N C13 cell retained high tumorigenicity ($TD_{50}=10^2$). We have known that the SV40 transformed SV AL/N cells, which were obtained from the same mass cell line from which the T AL/N cell arose, do not produce tumor even at 10^7 cell dose in the immunologically competent syngeneic mouse, because of the strong SV40 specific TSSA and TSTA. To study the balance between strong cellular tumorigenicity and superimposed SV40 induced antigenicity, the T AL/N clone 3 cells were "super"-transformed by SV40, and recloned. An SV40 T, TSSA and TSTA positive subclone, when injected into immunologically competent syngeneic mice, reproducibly gave T, TSSA and TSTA negative tumors, which were established as cell lines. These tumor cell lines were found, by the presence of a characteristic marker chromosome, to be direct descendants of the T, TSSA and TSTA positive subclone. Apparently, in vivo selection and antigenic recognition and rejection eliminated the (great majority) of SV40 T, TSSA and TSTA positive subclone cells, but allowed the growth of (rare) SV40 antigen negative revertant cells. Thus it became possible to select by in vivo passage for revertants negative for early SV40 function expression, but maintaining high tumorigenicity ($TD_{50}=10^2$). Studies by DNA reassociation kinetics indicate that the subclone cells contain about 2 copies of whole SV40 DNA, while the tumor lines (and several subclones obtained from the tumor lines) contain only about 0.5 copy number of predominantly late SV40 DNA, which is not transcribed to RNA.

The in vivo method allows for the first time selection in the immunologically competent host for revertant cells which lost the early (A) SV40 gene. No such selection occurred in the T cell deficient nude mice (the tumors were T, TSSA and TSTA positive). The experiments in the syngeneic mice open up a new kind of genetic, immunochemical and biochemical studies on the mechanism of (spontaneous) loss of a well characterizable gene (the A gene of SV40). Also, the in vivo selection method as exemplified above, when applied to tumorigenic cells which became malignant spontaneously or by some other means, and subsequently were transformed by immunogenic tumor viruses, may allow studies on genetic and cellular factors which control the balance between immunogenicity and tumorigenicity.

Significance to Biomedical Research and the Program of the National Cancer Institute:

Studies of cell surface biochemical changes which relate to acquisition of tumorigenic potential or to immunologic recognition and rejection in vivo are of interest, especially when they may lead to understanding of changes in molecular mechanism which appears to be reducible to heritable changes on control processes on the DNA level in the virally induced transformation of cells.

Proposed Course of the Project:

There will be an attempt to further clarify the relevance of the various changes in cell membrane biochemistry (glycoproteins, proteins, etc.) to spontaneous and to viral induced transformation of cells. We also plan to relate these changes to phenotypic changes in cell immunogenicity and in tumorigenicity in various syngeneic, allogeneic and nude mouse systems. We hope that the information and methodology developed in the mouse system shall be of use on human tumor cells.

Publications:

Luborsky, S. W., Chang, C., Pancake, S. J. and Mora, P. T.: Detergent solubilization and molecular weight estimation of tumor specific surface antigen from SV40 virus transformed cells. Biochem. Biophys. Res. Comm. 71: 990-996, 1976.

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Anderson, J. L., Chang, C., Mora, P. T. and Martin, R. G.: Expression and thermal stability of Simian Virus 40 tumor-specific transplantation antigen and tumor antigen in wild type- and tsA mutant transformed cells. J. Virol. 21: 459-467, 1977.

Anderson, J. L., Martin, R. G., Chang, C. and Mora, P. T.: Tumor-specific transplantation antigen is expressed during SV40 lytic infection with wild-type and tsA mutant viruses. Virol. 76: 254-262, 1977.

Anderson, J. L., Martin, R. G., Chang, C., Mora, P. T. and Livingston, D. M.: Nuclear preparations of SV40 transformed cells contain tumor-specific transplantation antigen activity. Virol. 76: 420-425, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05544-09 IP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Luborsky	Chemist	IP	DCBD	NCI
Other:	C. Chang	Visiting Fellow	IP	DCBD	NCI

COOPERATING UNITS (if any)

R. G. Martin, LMB, NIAMDD

LAB/BRANCH
Office of the Associate Director, Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study surface changes in transformed cells and the molecular mechanisms responsible for antigenic changes and/or the transformation to malignancy, either spontaneously or as induced by SV40 virus. To develop methods to isolate and purify cell surface membranes, and antigenic components such as SV40 virus specific surface antigens and tumor specific transplantation antigens; and to study their interrelationships.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: As part of a study of surface changes in transformed cells we wished to develop methods to isolate cell surface membranes, to compare those isolated from normal, spontaneously, and viral transformed cloned and mass cell lines; and to solubilize and characterize the antigens from SV40 transformed cells, and investigate their interrelationships.

Methods Employed: Tissue culture cell cultivation, infection with SV40 virus, cell cloning procedures, polyacrylamide gel electrophoresis, column chromatography, ultracentrifugation, various immunological assays to detect tumor antigens.

Major Findings:

An extraction and purification procedure was developed to obtain rapidly soluble membrane components from various cultured cells. These included AL/N mouse embryo cell lines such as normal and transformed cell lines. Solubilization was generally achieved using 0.5% Triton X-100 detergent in TBS, and was followed by ammonium sulfate fractionation. The extracts from SV40 transformed cells were assayed both in vitro,in inhibition of antibody mediated cytolysis (TSSA), in complement fixation tests for T antigen,and in vivo in immunization of mice against subsequent challenge with syngeneic SV40 transformed tumor cells (TSTA).

Using the detergent procedure we extracted the SV AL/N cells, and the similar spontaneously transformed T AL/N cells. We fractionated both extracts identically. The fraction which precipitated between 30-50% saturation with ammonium sulfate was put on a Sephadex G150 column. Only the extract from the SV AL/N cells had any TSSA activity. The TSSA activity was eluted both in the excluded volume and at a latter position, with apparent molecular weights of >200,000 and 25,000-50,000 respectively. A more direct determination of molecular size was obtained by centrifuging a combined 0-30% and 30-50% ammonium sulfate fraction though a 5-20% sucrose gradient. The sedimentation coefficient, s, of TSSA was estimated from the broad band of activity, at 2.5-4.5s, with the peak near 4s. The latter corresponds to an estimated molecular weight of 50,000-60,000. This was in reasonable agreement with the results of the gel exclusion experiments on Sephadex G150, especially as the sedimentation profile extended to the lower molecular weight region.

In a representative experiment, high yields (69%) of TSSA were obtained in whole cell extracts, and after ammonium sulfate fractionation (60%). Relatively high yields of TSSA were obtained from Sephadex G150 (16% and 9% of the whole cell activity) in the two peaks. Purification, however, was only obtained in the second peak, which had been retarded on the Sephadex G150, and showed a 2.6X increase in specific activity (units antigen/mg protein).

The detergent extracts from SV AL/N cells but not from T AL/N cells also exhibited TSTA activity against SV40 transformed mKSA tumor cell challenge. This TSTA was specific since it did not protect against meth-1-A tumor cells

challenge. The various TSSA active fractions described above were also active in TSTA assays. Thus, TSSA and TSTA co-purified through ammonia sulfate precipitation and Sephadex purification.

In order to understand the interrelationship between TSSA, TSTA and also the T antigen, we used extraction procedures originally developed by Livingston et al. These three antigens were fractionated through DEAE cellulose and phosphocellulose or DNA cellulose columns, and by sedimentation through a sucrose gradient. It was found that major amounts of these three antigens co-purified through the various procedures; furthermore, significant increases in specific activities were achieved. The SV40 TSTA binds to double stranded mammalian cell DNA, as does the T antigen. These results further support the hypothesis that SV40 T antigen and TSTA, although carrying different antigenic determinants, reside on the same polypeptide chain and likely are virally encoded.

We found a small amount of TSSA which did not co-purify with SV40 T antigen and TSTA. This, with other previous observations, indicates that the TSSA may also contain other antigens which are related to "transformation" but are not SV40 virus coded (embryonic antigens, etc.).

The binding of the T and TSTA activities to DNA is of particular interest, since it implies a possible role in control of DNA function, and hence in cell properties. We are the only laboratory at present which has available both rapid and quantitative fractionation as well as the assay procedures for the early SV40 antigens. These techniques thus can be used to compare and clarify the properties of these antigens and their possible dual role. In addition to acting as antigens, they may possibly act as elements in the establishment, control, or maintenance of the transformed state of a cell.

We have fractionated cells to obtain purified cell membrane, nuclear, microsomal, and cytoplasmic supernatant fractions. Our procedure is based upon those reported in the literature, and is adapted for our purposes to be simple, rapid and quantitative. When cells were labelled during growth with ^{35}S-methionine and fractionated, virtually 100% of the whole cell radioactivity was recovered in the various fractions, with the above 4 fractions of interest accounting for the major share. Radioimmuno-precipitation and autoradiography were then carried out to identify the nature, distribution, synthesis and processing of the early SV40 antigens.

Significance to Biomedical Research and the Program of the National Cancer Institute:

The properties of the cell surface, its chemical composition, structure and antigenic determinants exert important influences upon cell growth characteristics and the ability of certain cells to form tumors. Studies of cell surface biochemical changes which relate to acquisition and expression of tumorigenic potential or to immunologic recognition and rejection in vivo are therefore of particular interest. Since we have solubilized three SV40 specific antigens we can study the correlation of in vitro cytotoxicity and in vivo

transplantation-rejection assays as well as the molecular nature of these antigens and their possible interrelationships.

Proposed Course of the Project:

Extracts will be prepared from whole cells and from the isolated cell fractions, and assayed for SV40 antigens. Isolated antigens will be further characterized by ultracentrifugation as well as gel electrophoresis, to determine more precise values for their molecular weights. Purified antigen preparations will be used to raise antibody for the preparation of affinity columns to more rapidly and quantitatively prepare SV40 antigens for further study. In the light of their co-purification, we will attempt to understand the interrelationships of these early SV40 antigens by investigating early events leading to their synthesis and possible processing. The distribution of these antigens in the cell will be investigated in various cell lines. We will attempt to better understand the relevance of the various changes in cell membrane biochemistry and immunochemistry to spontaneous and to viral induced transformation of cells.

Publications:

Luborsky, S. W., Chang, C., Pancake, S. J. and Mora, P. T.: Molecular weight estimation of detergent solubilized tumor specific surface antigen from SV40 virus transformed cells. Biochem. Biophys. Res. Comm. 71: 990-996, 1976.

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Annual Report of the Laboratory of Immunodiagnosis, National Cancer Institute

July 1, 1976 - September 30, 1977

This report summarizes the activities of the Laboratory of Immunodiagnosis for its second full year, since its initiation in December, 1974, and its first year without major changes in senior personnel. The only significant change in senior staff has been the addition of Dr. James L. McCoy to the Laboratory as an Expert Consultant. Each of the areas of research in this laboratory has been highly productive during the past year: There have been extensive efforts toward characterization of natural killer (NK) cells in mice, rats and humans, and a demonstration of a close similarity between NK cells and K cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC). A lack of complete H-2 restriction in T cell-mediated anti-tumor immunity has been documented. A human lung cancer-associated antigen has been purified and isolated, and this may be useful in the diagnosis of lung cancer. There has been very substantial progress in the development of assays of cell-mediated anti-tumor immunity, particularly migration inhibition assays, and the role of macrophages in these reactions has been carefully examined. Much information has been analyzed on the relationship of clinical course of disease in cancer patients to the results of various assays of immunologic competence and anti-tumor immunity.

In addition to the research activities of the Laboratory, which are summarized in more depth below, Drs. Herberman and McIntire have continued to be extensively involved in the National Cancer Program activities related to immunodiagnosis of cancer. Both serve on the Committee on Immunodiagnosis of Cancer and other contract review committees. Dr. Herberman is the main focus within the National Cancer Institute for problems related to immunodiagnosis, and he has been particularly involved with the evaluation of many potentially useful serum immunodiagnostic tests, by the use of coded serum panels provided to investigators from the NCI-Mayo Clinic Serum Bank.

Because of the limited space and technical staff which have been available to the Laboratory, its activities are substantially augmented by support contracts with Litton-Bionetics, Inc., Kensington, Md., and with George Washington University Medical Center, Washington, D.C. These permit more extensive studies of *in vivo* tumor immunity in experimental mice and rats, *in vitro* assays of immunity of cancer patients by established procedures, and large-scale separations of human tumor-associated antigens. These support contract projects are performed in very close association with the intramural activities of the Laboratory.

The research activities of the Laboratory of Immunodiagnosis can be divided into three main areas: Cell-mediated immunity studies in animal tumor systems, cell-mediated immunity studies in man, and immunochemical studies of tumor-associated antigens. The main emphasis in each of these areas is on the application of the research information to the diagnosis of cancer and monitoring of disease in tumor-bearing individuals.

Some highlights of achievements in the past year:

I. Cell-Mediated Immunity Studies in Animal Tumor Systems.

Extensive studies have been continued on the cell-mediated immune response of C57BL/6 mice to inoculation with murine sarcoma virus (MSV). Earlier studies from this laboratory have shown that cytotoxic reactivity of spleen cells from C57BL/6 mice injected with MSV 14 days earlier was mediated by T lymphocytes and was directed against endogenous type C virus antigen, designated MEV-SA-1. When cytotoxicity was tested in a 4-hr ^{51}Cr release cytotoxicity assay, there appeared to be an association between the major histocompatibility locus (MHC) and the cytotoxic reactivity, since syngeneic targets could be lysed efficiently while allogeneic targets were not. However, when the cytotoxicity assay was increased to 18 hours, reactivity was observed against targets which were dissimilar at the MHC. Similar studies were performed in BALB/c mice injected with MSV. Reactivity was also detected by these effectors against syngeneic and allogeneic tumor targets carrying the appropriate antigens. However, the magnitude of the reactivity against the allogeneic target was lower than that found using spleen cells from MSV-immune C57BL/6 mice. Hence in our experimental system we have found H-2 preference rather than H-2 restriction. Other studies have provided additional information on the role of the MHC (or lack thereof) in the interaction between T lymphocytes and tumor-associated antigens. Animals that have been immunized against MSV can undergo a secondary cytotoxic response against a syngeneic target when they are challenged in vivo with either syngeneic or allogeneic tumor cells. Similarly, a secondary cytotoxic response can be elicited in vitro against a syngeneic target using as stimulators either syngeneic or allogeneic tumor cells. Monolayers of syngeneic tumor cells are capable of adsorbing out the cytotoxic reactivity against syngeneic and allogeneic tumor targets that are carrying the appropriate antigens. Finally, using an entirely different measure of T cell function, production of migration inhibition factor (MIF), we have been able to show that interaction between T cells and tumor-associated antigens can occur even when the MHC on the stimulating cell is different from that on the sensitizing cells. C57BL/6 immune T cells ($H\text{-}2^b$) can be stimulated to produce MIF by BALB/c ($H\text{-}2^d$) or A/J ($H\text{-}2^a$) tumor cells as well as by C57BL/6 tumor cells. In vivo protection experiments confirmed our previous in vitro data. When (C57BL/6 x BALB/c)F1 mice were immunized with the tumor line LSTRA of BALB/c origin, they resisted challenge with the same BALB/c tumor, and also with MBL-2 tumor cells of the other parental H-2 type, C57BL/6.

As described last year, lymphoid cells from many normal mice and rats, of a variety of inbred strains, were found to have reactivity, in the 4-hour ^{51}Cr release cytotoxicity assay, against several syngeneic and allogeneic tumors. During the past year, we have extended some of the findings made in mouse to other species and have found that there are many similarities in natural killer (NK) cells among various species. Most of the initial studies with mouse NK cells in a short-term ^{51}Cr release assay were against lymphoma target cells and antigenic specificities associated with endogenous type C viruses. However, our recent studies have indicated a considerably broader range of reactivity. Many mouse carcinomas and sarcomas have been found to

be susceptible to NK activity, and there has been some activity against normal thymus cells, bone marrow cells and macrophages. In addition, the cytotoxicity has not been restricted to cells from the same species. Mouse, rat and human NK cells have been found to have considerable reactivity against some, but not all, heterologous target cells. From direct testing and inhibition studies, it appears that NK cells can react against a variety of antigenic specificities. Detailed studies have been formed on the nature of NK cells. It was initially thought that mouse and rat NK cells were lymphocytes which lacked any identifiable cell surface markers. However, since human NK cells were found to have Fc receptors and low affinity receptors for sheep erythrocytes, it was of interest to closely reexamine the rodent NK cells for Fc receptors and for T cell markers. These studies have indicated that at least the majority of rodent NK cells have receptors for the Fc portion of IgG. To more closely examine the possible T cell derivation of mouse NK cells, repeated studies have been performed with antisera to the Thy 1.2 antigen. Treatment of NK cells from nude mice, or from older conventional mice with NK activity boosted by lymphocytic choriomeningitis virus (LCMV), resulted in a substantial reduction of NK activity.

Spontaneous expression of NK reactivity in mice and rats is quite transient, being detectable only at 5-10 weeks of age. However, it has been possible to augment the levels of NK reactivity in young mice, or to cause a reappearance of NK reactivity in older mice, by inoculation of cells bearing the relevant antigens or nonspecifically by inoculation of LCMV, other mouse viruses, BCG or Corynebacterium parvum (C. parvum). All of these treatments produced a peak in cytotoxicity at 3 days, which was detectable in all of the lymphoid organs, except the thymus, and in the bone marrow. Recent studies indicate that C. parvum and some other agents can similarly cause high increases in NK activity in rats. The effector cells mediating the boosted cytotoxicity had the same characteristics as those of NK cells in unstimulated animals.

Using a long-term (18-hr) ^{51}Cr release cytotoxicity assay, we have performed parallel studies on the distribution and nature of the effector cells mediating NK and ADCC activity. Both activities were found to have the same age distribution. In newborn and weanling animals the reactivity was usually low. NK and ADCC cytotoxicity showed peak reactivity at about 5-8 weeks of age and then there was a decrease after 10 weeks of age. Among different strains of conventional, thymus-bearing mice a good correlation was seen in tests for NK and ADCC activities against the same target cell (RBL-5 tissue culture). CBA and C3H/f mice had high levels of reactivity, C57BL/6 and BALB/c had intermediate levels and SJL had low levels. As with NK reactivity, young nude mice with random bred, BALB/c or C57BL/6 genetic backgrounds had high levels of ADCC activity. The NK and K cell reactivities were not restricted to the spleen but were present in other lymphoid organs as well. Effector cells from the spleen and peritoneal cavity were shown to be very reactive; cells pooled from axillary, inguinal and mesenteric lymph nodes or cells from bone marrow had lower, but significantly positive reactivity. Thymocytes were found invariably to be nonreactive in both tests. Several different *in vitro* and *in vivo* manipulations have been found to influence the level of NK and ADCC activity in a similar fashion. Inoculation of older mice (that have very low levels of both activities) with LCMV or

Corynebacterium parvum results in an increase in ADCC activity as well as NK activity. On the other hand, both reactivities were found to be depressed in MSV tumor-bearing nude mice. The sensitivity of NK and K cells to irradiation in vitro was similar. Doses of 500 R or less had little effect; however, with increasing doses of irradiation there was a corresponding decrease in both NK and ADCC activity. The effector cells in both tests seemed to have similar surface markers and other characteristics. The NK and K cells were nonphagocytic and nonadherent. Adsorption of spleen cells on EA monolayers to remove Fc receptor-bearing cells resulted in a moderate decrease in both K and NK cell reactivities. There have been some consistent differences between the characteristics of the NK and K cells. NK activity in a short-term assay was trypsin sensitive. However, NK activity in the long-term assay and ADCC were not affected by trypsin when effector cells from CBA mice were employed, but when effector cells from nude mice were used, NK activity decreased while ADCC did not. In addition, NK activity was found to be relatively more labile upon short-term incubation at 37°C than was ADCC. These findings, plus the many similarities between mouse NK cells and K cells for antibody-dependent cytotoxicity against mouse tumor target cells, have raised the possibility that natural cytotoxicity is mediated by K cells armed in vivo with natural antibodies, via their Fc receptors. Studies are in progress to carefully investigate this hypothesis.

An increasing amount of data indicate that NK cells may play an important role in in vivo resistance to tumor growth. A number of mouse lymphomas, carcinomas, sarcomas and the B16 melanoma have been found to grow poorly in nude mice, which have high levels of NK activity. Each of these tumors has been shown to be sensitive to NK activity. Some schedules of inoculation of mice with LCMV to boost their NK activity have resulted in increased resistance to tumor growth. In addition to a role in tumor resistance, we have performed studies to determine the possible role of NK cells in resistance to bone marrow transplants. There are a number of analogies between these two phenomena: development at about 3 weeks of age, sensitivity to cyclophosphamide and relative resistance to x-irradiation. In addition, our findings that some bone marrow cells are sensitive to NK activity, that NK activity is detectable in the bone marrow, and that inoculation of allogeneic bone marrow cells can boost NK activity are suggestive of a role of NK in bone marrow resistance.

Rats immune to the Gross leukemia-virus-induced lymphoma, (C58NT)D, have been shown to have lymphoproliferative responses to tumor cells bearing the relevant antigens. However, these responses were only detectable between 15-60 days in rats regressing their tumors and were completely undetectable in rats bearing progressively growing tumors. It has been shown that T lymphocytes with the ability to undergo a proliferative response to (C58NT)D were present at other times in rats inoculated with the tumor, but that suppressor macrophages inhibited the responses. The spleens and peritoneal exudates of even normal rats and immune rats not bearing tumors were found to have suppressor macrophage activity. Removal of these suppressor cells by rayon adherence columns or by treatment with carbonyl iron plus magnet resulted in the ability to demonstrate otherwise undetectable reactivity. The presence of these suppressor cells in normal rat spleens was shown to account for the difficulty

in demonstrating their responsiveness in mixed leukocyte cultures. Furthermore, by removal of these suppressor cells, we were able to re-examine the kinetics of the proliferative response of rats inoculated with (C58NT)D. Reactivity was detectable within 10-12 days, reached peak levels between 30-60 days but remained detectable for well over one year. This persistent reactivity was compatible with our previous observations of long-lasting resistance of immunized rats to challenge with tumor cells. Recent studies have indicated that other aspects of the cellular immune response against tumor antigens are also quite persistent. The ability to generate cytotoxicity upon re-exposure to tumor cells in vitro, production of migration inhibitory factor, and systemic adoptive transfer of immunity were all found to be long-lasting. These findings make it more likely that some or all of these responses play a significant role in in vivo resistance against tumor growth.

Because of the persistent ability of MSV immune lymphocytes to develop a strong secondary cytotoxic response, we have examined the requirement for proliferation in this response. Suicide experiments with high specific activity ^{3}H-thymidine have shown that cell proliferation is required for the generation of secondary cytotoxicity from the spleens of mice infected with MSV.

Macrophages recovered from the spleen, peritoneal cavity and the tumor of murine sarcoma virus (MSV)-induced tumor-bearing mice strongly inhibited the proliferation of RBL-5 cells in a 48-hour growth inhibition assay (GIA). In addition, these macrophages were shown to have cytolytic activity in an 18-hour ^{51}Cr release assay. The highest cytolytic activity was seen with macrophages from the disaggregated tumor. In the MSV tumors, about 50-60% of the cells were macrophages while another 30-40% were T cells. These observations support the hypothesis that cytolytic macrophages may play a role in the spontaneous regression of the tumor, in addition to the important role of the immune T cells.

Extensive studies also have been performed with the migration inhibition assays, to determine their relationship to in vivo anti-tumor immunity. Spleen cells from animals immunized with MSV, obtained 9-19 days after inoculation of virus, when incubated in culture with antigen to which they had been sensitized produce migration inhibition factor (MIF). Since we have shown that MIF production in the MSV system is T cell dependent, it was of interest to determine which cell population produced MIF and which populations cooperate in this immune function. Using populations of immune spleen cells depleted of macrophages by different techniques, we found that such purified spleen cells were unable to produce MIF when stimulated by soluble antigen. However, the ability of these cells to produce MIF could be restored by the addition of normal, syngeneic macrophages which had been previously pulsed with the soluble antigen. Moreover, we observed that the macrophage-lymphocyte interaction was under the control of genes mapping in the MHC (H-2). Additional studies with two different strains of animals and with congenic resistant strains of the C57BL/10 mice have confirmed these findings and indicate that this cooperative function maps at either the K or I region within the MHC. By contrast, using intact tumor cells as a source of antigen instead of soluble tumor extracts, macrophages were not required in order to stimulate MIF production. Furthermore, direct lymphocyte-tumor cell interactions were not under H-2 restriction since both syngeneic and allogeneic tumor cells

could be used as stimulators. Using a 1 g velocity sedimentation technique we found that two different subpopulations of T lymphocytes were responsible for the production of MIF by the different antigens. One population, with a sedimentation velocity of 2.5-3.5 mm/hr, released MIF only when stimulated with soluble antigen in the presence of macrophages. The other population which had a sedimentation velocity of 4.5-5.5 mm/hr released MIF only in the presence of intact tumor cells, without a detectable requirement for macrophages. Moreover, the fraction which produced MIF in the presence of intact tumor cells was the only population which was cytotoxic in the 4-hr ^{51}Cr release cytotoxicity assay. Finally, we have found that macrophages can interfere with the production of MIF in a number of circumstances. When immune spleen cells which had been depleted of macrophages were mixed with increasing percentages of macrophages up to 10%, MIF production increased in response to soluble antigen. Addition of 15-20% macrophages actually decreased the MIF production. Furthermore, spleen cells from animals that had been injected with MSV 14 days earlier sometimes responded poorly to stimulation with intact tumor cells. By removing the macrophages, these spleen cells then produced high levels of MIF. When the spleen cells were stimulated with soluble antigen, removal of macrophages from the population and addition of 5-10% normal peritoneal macrophages often resulted in a better response to soluble antigen.

Macrophage migration inhibition assays, using the direct agarose microdroplet method, were used to monitor antigenic activity of preparations of mouse SV40-induced tumor cells and of plasmacytomas. The antigen preparations included cell-free crude membranes, papain-solubilized and NP40 detergent-solubilized membrane extracts from mKSA tumor cells. The assay was extremely sensitive and could detect migration inhibition reactivity with all three types of antigenic preparations with concentrations as low as 250 picograms protein/ml. The reactivities were quite reproducible and specific for the homologous tumor antigens. The results demonstrated the usefulness of the assay in rapidly detecting small concentrations of partially purified TAA preparations using small numbers of immune cells.

We were interested in seeing whether the agarose microdroplet migration inhibition assay of LMI could be used to measure reactivity against TAA and PPD using PBL of guinea pigs bearing syngeneic line 10 hepatocarcinomas or cured of tumors with BCG therapy. LMI assays were also performed in further investigating the possible antigen cross-reactivity between line 10 tumor and BCG. We successfully demonstrated cell-mediated reactivity to line 10 tumor antigens or PPD with the agarose microdroplet LMI assay using peripheral leukocytes from line 10 tumor-bearing animals or animals cured of tumor with BCG. The assay was quite sensitive in that nanogram and picogram concentrations of the crude line 10 antigen and PPD were capable of eliciting strong LMI reactivity in some immune animals. Our LMI data were also strongly suggestive of a cross-reactive antigen between line 10 tumor and BCG. Animals exposed to only line 10 reacted with PPD and others sensitized with only BCG reacted with line 10 antigen. There was a significant correlation between line 10 and PPD antigen reactivity in simultaneous LMI tests with animals sensitized with only BCG, whereas no significant correlation between PPD and syngeneic but antigenically unrelated L2C antigens was observed with these animals in simultaneous tests. Since we were successful in demonstrating

reactivity to PPD and TAA of line 10 hepatocarcinoma cells by immune guinea pigs in agarose microdroplet LMI assays, we used the assay to longitudinally monitor reactivity against the antigens in guinea pigs either cured of line 10 tumors with BCG or line 10 tumor-bearing animals. These results indicated that LMI may be predictive of efficacy within a randomized protocol.

We have recently initiated migration inhibition studies of cellular immunity against TAA and mouse mammary tumor virus and gp52 in a syngeneic mammary virus tumor model in C3H mice. Early reports with transplantation rejection studies suggested that C3H (C3H+) mice congenitally infected with mammary tumor virus via milk transmission were immunologically tolerant to antigens of the mammary tumors, whereas C3H (C3H-) mice foster nursed and negative for the virus were immunologically reactive against such antigens. We have been inoculating C3H+ and C3H- mice with a transplantable C3H+ mammary tumor (MT-2) which grows progressively. Spleen cells of tumor-bearing animals were harvested at various times after tumor inoculation and in an indirect assay tested for MIF production in response to a 3M KCl extract of MT-2, mouse mammary tumor virus and gp52. Both C3H+ and C3H- mice bearing the MT-2 tumor developed reactivity against the tumor and viral antigens, and these results demonstrate that C3H+ mice are not tolerant to either antigens of a mammary tumor or to antigens of mouse mammary tumor virus. Further, they suggested that an eclipse of immunity as detected by the in vitro assay can occur in large tumor-bearing C3H+ mice, a phenomenon not yet seen in C3H- mice.

II. Studies of Cell-Mediated Immunity in Man.

Since we recognized the importance of natural reactivity in various cytotoxicity assays, we have studied in detail the characteristics of the human natural effector cells and their relationship to other effector mechanisms. As in the rodent studies, we have particularly examined the relationship between NK cells active against the myeloid cell line K-562 and K cells mediating ADCC against antibody-coated Chang cells.

NK cells were characterized as lymphocytes having an Fc receptor (FcR), and in contrast to previous reports, no complement receptor (C3R). The NK cells also demonstrated low-affinity binding to sheep erythrocytes (sE), and therefore appeared to belong to a subpopulation of T cells.

K cells active in ADCC against Chang target cells represent a well-characterized FcR-positive lymphocyte population. We showed that NK cells and K cells could not be physically separated from each other by several different techniques, and raised the possibility that they were within the same lymphocyte subpopulation. In particular, the sE-rosetting procedures used revealed that the majority of K cells were also low-affinity binding sE-RFC (rosette-forming cells).

Although they remained physically inseparable, there were noticeable differences between the NK and K cells and/or their mechanisms of action. Trypsin treatment of the effector cells abolished NK, and left ADCC intact; also, Protein A abolished ADCC, and left NK intact. These data were consistent with the hypothesis that NK cells could simply be K cells that were "armed" in vivo with natural antibodies against K-562, and various other cell lines.

Additional evidence for similarities between NK and K cells was obtained from experiments in which lymphocytes were cultivated, either alone or in mixed leukocyte cultures (MLC), and the indirect cytotoxicity was tested in CRA against K-562, antibody-coated Chang cells and allogeneic PHA blasts. It became quite clear that cytotoxic activities, with specificity for the NK and ADCC targets, were maintained in cultures of lymphocytes alone, and were also present in MLC reactions. The source of serum was quite important, with cytotoxicity developing in fetal bovine serum (FBS) much better than in human S. Of interest was our observation that high levels of cytotoxicity were also obtained even in human serum, when various mitogens and various preparation of mumps virus were used as stimulating agents. The data also indicated that the cytotoxicity against K-562 was mediated by FcR-positive effector cells, whereas the cytotoxicity generated in MLC against PHA blasts was mediated by FcR-negative effector cells.

The broad cytotoxicity mediated by lymphocytes of healthy donors and patients alike against a variety of tumor-derived target cell lines has often been called nonspecific. We have analyzed this by the competitive inhibition assay, using unlabeled target cells as inhibitors of CRA. We found that the NK activity defined in the K-562 system represented the activity of cells with a broad but demonstrable specificity. A majority of tumor cell lines used as inhibitors of the CRA against K-562 target cells were effective. Among them, one could distinguish one breast cancer-derived cell line, G-11, that inhibited particularly well. This line was also susceptible to NK, when used as target. Fresh tumor cells (lung, breast) were also inhibitory. Noticeably, a few B cell lines, ALL and CML blasts, and fresh lymphocytes or PHA-blasts failed completely to inhibit.

It was possible to use what we learned about the NK cells and their specificity in attempts to discriminate this NK activity from disease-related cytotoxicity. First, we compared the levels of cytotoxicity for several groups of donors against K-562, G-11 and MCF-7, a well-characterized breast cancer cell line. Paired comparisons were made and donors with increased cytotoxicity against G-11 and MCF-7 relative to NK against K-562 were identified. A significantly higher frequency of increased reactivity against G-11 was observed with patients with breast cancer but also with normal male donors and patients with benign breast disease. A more breast cancer related pattern of increased reactivity was obtained with the MCF-7 cell line. Patients with other cancers also had more often relatively increased reactivity against G-11, but not so against MCF-7. The study singled out MCF-7 as a good target for reactivity of breast cancer patients with little susceptibility to NK, whereas G-11 was more susceptible to NK.

In a further effort to dissect out the cytotoxic effector cells in MPBL of normals and patients, use was made of the presence of FcR on NK cells active against K-562. Immunoadsorption experiments were carried out on sEA monolayers, and cytotoxicity was then tested using the nonadherent fraction of lymphocytes. This procedure almost completely eliminated cytotoxicity against K-562 and G-11, and also diminished the cytotoxicity against lung or melanoma lines. However, a few individuals were identified whose MPBL obviously had an FcR-negative population that was responsible for cytotoxicity against the cell lines other than K-562.

During the past year, extensive studies on the immunologic reactivity of separated lymphoid populations have been performed, in an attempt to identify the cells responsible for immunologic reactivity against tumor antigens. During these studies, monocytes have been found to be required for various responses of lymphocytes. By the passage of MPBL over Sephadex G-10 columns it was possible to reduce the monocytes to below 0.1%. This was accompanied by a substantial decrease in the proliferative responses of lymphocytes to the mitogen Con A and to antigens. Also, we found that separated lymphocytes forming rosettes with sheep erythrocytes (sE-RFC) were almost completely devoid of monocytes, and this abolished the proliferative responses of these cells to Con A. Adequate responses were obtained by addition of small numbers of monocytes.

We continued to use sE rosetting techniques and flotation on Ficoll-Hypaque for the purification of T cells in the sE-RFC pellet. A protocol with two centrifugation steps resulted in excellent purity of the sE-RFC, and in a non-sE-RFC interface population. Under these circumstances, and given adequate numbers of monocytes, the sE-RFC had proliferative responses to PHA, Con A, and antigens like PPD and mumps, whereas cells from the interface had no such responses. Protocols have been designed for the application of these techniques to tests of cell-mediated tumor immunity, namely to the mixed lymphocyte tumor interaction (MLTI), the production of leukocyte migration inhibition factor (LIF), or cytotoxicity experiments performed with patients' MPBL.

Distinct subpopulations of T cells were identified on the basis of their affinity for sE. When the sE-RFC assay was done at 8°C, with an optimal ratio of sE to MPBL, the total number of sE-RFC was identified (77 $\pm$ 4%). However, the same technique performed at 29°C, with a lower ratio of sE to MPBL, identified a subpopulation of high-affinity sE-RFC (54 $\pm$ 5%). Low-affinity sE-RFC (23 $\pm$ 3%) could be sedimented through Ficoll-Hypaque gradients after rosetting at 8°C the cells which had not sedimented during an earlier sE separation done at 29°C. These low-affinity sE-RFC have an FcR, and they were shown to represent the vast majority of FcR-positive lymphocytes in MPBL. They were similarly prominent in spleen cells, but not in lymph nodes; they were absent from the thymus, as assessed both by the sE-RFC characteristics and the presence of an FcR. There was an excellent correlation between the presence of these FcR-positive, low-affinity sE-binding cells in lymphocyte preparations, and the ability of such populations to mediate NK or ADCC. Thus, lymph node cells and thymus cells had little or no activity, with regard to NK and ADCC. Individuals with lower numbers of FcR-positive or low-affinity sE-RFC were less active in NK and ADCC assays. In contrast, evidence in this and other laboratories has suggested that precursors and effector cells for proliferative and cytotoxic responses in MLC were in the high-affinity, FcR-negative subpopulation of T cells.

During the period under review, we had an opportunity to study cultured T cells. These cultures could be initiated with MPBL from most normal donors with the action of conditioned media from allogeneic PHA-stimulated lymphocytes. The presence of 20% of the conditioned media (CM) was a prerequisite for the growth of the T cells, at all passage levels. When taken off the conditioned medium, cells remained viable only for a few days, and stopped

proliferating. These T cells were not transformed, did not carry the Epstein-Barr virus nuclear antigen and had a normal karyotype. One of these cultures was 11 months old. Similar T cell cultures could be initiated from human bone marrows and spleens. Our studies have shown the cultures to be virtually pure T cells, with no evidence for B cells. These cells were >98% sE-RFC, with 70% showing high-affinity binding when grown on the conditioned media. However, when taken off the conditioned media, >90% of the cells demonstrated high-affinity binding to sE-RFC. Only a small proportion (0-4%) had detectable FcR, and there were detectable B cells. A growth promoting factor was found in the CM that was separable from contaminating PHA, and this is now being characterized. The cultured T cells displayed proliferative responses to allo-antigens in MLC, but their responses to the T cell mitogens PHA, Con A and PWM were lost after a few passages.

A considerable emphasis has been placed on evaluation of cellular immune competence in cancer patients. This has included the enumeration of the percent of T cells in the peripheral blood by rosette formation with sheep erythrocytes. The test was performed under suboptimal conditions, at 29°C, as well as under optimal conditions at 8°C. The technique has been very well standardized, and this allowed considerable testing of cancer patients, including more longitudinal follow-up studies. In our study of serial monitoring of post-operative lung cancer patients, the rosette results have shown much promise in their clinical correlations, and particularly in their ability to predict recurrence of disease. Thus 26/36 patients with recurring disease had decreased "high-affinity" sE-RFC values a mean of 3 months before clinical signs of recurrence. This contrasts with decreased high-affinity E-RFC in only 2/20 patients without evidence of further disease. With breast cancer patients we found that those on adjuvant chemotherapy had considerably depressed values of "high-affinity" sE-RFC, but there was a strong trend towards restoration in those patients who also received Corynebacterium parvum as immunotherapy.

Cell-mediated proliferative responses to the mitogens Con A and PHA, and to alloantigens in MLC, were often severely depressed in lung cancer patients, and with lesser frequency in breast cancer. We worked out the methodological problems related to an accurate assessment of immunodepression with these tests, and defined a relative proliferation index, based on a comparison of the activity of each patient with the values for three or more normal donors tested the same day. Con A (40%), PHA (50%) and MLC (47%) responses were depressed in lung cancer patients, and these responses were also depressed in breast cancer (19%, 31%, 36%, respectively). There was some correlation of the depression with the stage of the disease, and a preliminary analysis of the data suggested its usefulness for predicting recurrence. Studies were initiated recently to determine possible mechanisms for the observed depression. About one half of the cancer patients with immunodepression were shown to have suppressor cells active on normal third party lymphocytes. In some instances, the suppressor cells appeared to be monocytes, as was reported in studies with rodent tumors. In other instances, the suppressor cells were nonadherent, and need to be characterized further. With other depressed patients, there was no evidence for suppressor cells and this may reflect an intrinsic defect or decreased numbers of responsive lymphocytes.

We measured delayed hypersensitivity reactions of patients to Sephadex G-200 and polyacrylamide gel electrophoresis (PAGE) purified soluble fractions of tumor cell extracts, prepared by Dr. A. Hollinshead. The major emphasis during the past year has been to test large numbers of cancer patients, with large batches of purified extracts prepared from standardized sources, mainly tissue culture cell lines shown to be antigenic in in vitro assays. The main concern has been to verify, in this large-scale testing, the specificity of these skin test antigens, which had been suggested by earlier studies with small amounts of materials and small numbers of patients. Some PAGE fractions from the MCF-7 tissue culture line have been shown to give positive skin tests in many breast cancer patients (16/38), and only in a small proportion of patients with other types of cancer. Therefore, we have been able to confirm the breast cancer-associated specificity of this material, and we are currently using it for serial tests of all patients on our clinical breast cancer protocol to determine the relationship of reactivity to clinical status and response to therapy.

Leukocyte migration inhibition (LMI) appears so far to be the most potent in vitro tool in our laboratory to detect TAA on human tumors and tumor-derived cell lines. Most of our protocols included a thorough, longitudinal investigation of LMI reactivity to various tumor extracts. Patients with lung cancer were tested with 3M KCl extracts of a tissue cultured squamous cell lung tumor (CaLu-1) and a fresh pleural effusion (7661) from a patient with pulmonary adenocarcinoma. A total of 40/67 (60%) lung cancer patients reacted to 7661 as compared to 4/40 (10%) normal donors. A similar reactivity (21/38 lung cancer patients and 1/16 normal donors) was obtained with the CaLu extract. There was no histological restriction in LMI reactivity with these two extracts. Patients with different stages of disease had similar degrees of reactivity and patients were also positive during relapse and within a month of death. However, the incidence of reactivity appeared to decrease substantially in patients who remained free of disease.

A high proportion of breast cancer patients were shown to react in LMI with MCF-7 tissue culture cells derived from breast carcinoma. The results indicate that the MCF-7 cells possess a tumor-associated antigen to which many breast carcinoma patients are sensitized. As in the lung cancer studies, patients who remained free of recurrent disease had substantially lower reactivity than did patients who recurred.

Because of the potential etiologic and diagnostic implications of a potential specific immunologic relationship between human breast cancers and mouse mammary tumor virus, direct migration inhibition and lymphocyte proliferation assays were conducted with leukocytes from breast carcinoma, benign breast disease, other cancer patients, and normal donors using mouse mammary tumor virus (MMTV), its major glycoprotein (gp52), murine leukemia virus (MuLV) glycoproteins (gp69/71), a normal mouse H_2 glycoprotein and 2 human glycoproteins and fetuin (a glycoprotein of fetal bovine serum) as antigens. Results suggest that many breast carcinoma and benign breast disease patients have LMI hyper-reactivity against many glycoproteins including those of MMTV and MuLV.

Using crude antigen preparations obtained by 3M KCl extractions of fresh Ewing's sarcoma or of tissue culture cells derived from a pleural effusion of Ewing's sarcoma patients, assays were performed with these patients' leukocytes, patients with cancer of other sites, and normal donors. The results indicate that many patients with Ewing's sarcoma have cellular immunity toward TAA on Ewing's sarcomas.

We wanted to document that the migration inhibition reactivity against tumor antigens was mediated by the lymphokine, LIF. Therefore, indirect migration inhibition tests were performed with polymorphonuclear cells and supernatants generated in cultures of mononuclear cells with either MCF-7, the major glycoprotein (gp52) of mouse mammary tumor virus, or a lung cancer extract, 7661. Agarose microdroplet assays were used to test the supernatants in order to demonstrate that this assay could measure LIF activity. Greater than 50% of the breast cancer patients tested generated reactive supernatants in response to both the 3M KCl extract of MCF-7 and the gp52 antigens. A similar frequency of positive results was obtained when mononuclear cells of lung cancer patients were incubated with the 3M KCl extract of 7661. No appreciable reactivity was seen with supernatants generated by these 3 antigens when incubated with mononuclear cells of normal donors. The activity of the supernatants appeared to be due to the lymphokine, LIF, since fractionation of the supernatants on Sephadex G-200 showed the active materials to have a molecular weight in the range of LIF (69,000). Thus, the results indicate that these antigenic materials induce the specific production of the lymphokine, LIF, by cancer patients.

Lung and breast cancer patients were tested for the proliferative responses of their MPBL to autologous tumor material. This protocol suggests that reactivity would measure reactions against TAA. Sixty percent of our tests in lung carcinoma and 40% of our tests in breast carcinoma resulted in clearcut proliferation. Low concentrations of hypotonic membrane preparations worked particularly well in this system, and they have allowed serial monitoring studies. We observed that breast cancer patients would usually lose their reactivity after X-irradiation therapy. The assessments of the production of LIF in these cultures, and of the generation of cytotoxic effector cells, have been incorporated in these protocols, to allow further evaluation of the MPBL reactivity towards tumor-derived extracts. We found that cells from several breast cancer patients, when cultured with their hypotonic membrane preparation, would develop increased levels of selective cytotoxicity against the MCF-7 cell line. In addition, about 80% of the patients responded to their autologous tumor antigens by detectable production of LIF.

We have been assessing the possible benefits of post-operative adjuvant immunotherapy with BCG and allogeneic tumor cells, or BCG alone in a randomized controlled trial with lung cancer patients. It was quite clear that patients with stage III, local residual tumor burden accrued no benefit from the immunotherapy and this part of the study was discontinued. In contrast, our results to date with patients with stages I and II indicate a trend toward prolonged disease-free interval in the groups receiving immunotherapy.

III. Immunochemical and Immunodiagnostic Studies of Tumor-Associated Antigens.

We are continuing to perform assays for alpha fetoprotein (AFP) and human chorionic gonadotropin (HCG) to provide evidence that these marker proteins are of great value for following therapy in hepatocellular carcinoma, germ cell carcinomas of the testis and pancreatic carcinoma. The combined use of AFP and HCG has been demonstrated to provide an accurate tumor marker for about 90% of patients with non-seminomatous testicular germ cell tumors. The two markers provide evidence of sub-clinical tumor that can reduce the error in staging by clinical means alone. In several collaborative studies utilizing combined modalities of therapy the regular quantitation of AFP and HCG has become an extremely important aspect of the evaluation of therapy. Consecutive rising values of either invariably have meant that the tumor has become resistant to the present therapy or somehow has started to grow and will be clinically evident within a few months -- it signals the time to consider a change in therapy.

During the past year the assay for AFP has been improved for greater sensitivity. The mean normal adult level is now 3.5 ng/ml and the assay sensitivity is 500 pgm/ml (formerly 20 ng/ml and 5.5 ng/ml, respectively).

Studies on the early detection of hepatocellular carcinoma in monkeys treated with chemical carcinogens are continuing. The AFP serum level is clearly elevated many months before the tumor is clinically detectable and increases with increasing tumor size. During the past year we have developed a sensitive radioimmunoassay (RIA) for monkey AFP to replace the previous modification of the human AFP assay. In vitro synthesis of AFP in 5 different carcinogen-induced monkey hepatomas was demonstrated by both specific incorporation of ^{14}C amino acids and AFP quantitation in tissue culture media in 4 of the tumors. The degree of tumor cell differentiation showed an inverse correlation with the synthetic rate of AFP. Extensive studies of AFP turnover in monkeys have also been performed.

Specific antiserum to AFP and HCG has been used together with a second antibody conjugated to horseradish peroxidase to identify the cells associated with each of these markers. HCG is identified with syncytiotrophoblastic giant cells and choriocarcinoma while AFP is found in embryonal carcinoma cells and endodermal sinus tumor. This procedure has allowed the retrospective identification of tumor markers, at a time when serum may be unavailable.

Studies of a human lung tumor-associated antigen (LTAA) have continued. Using ion exchange and affinity chromatography, and polyacrylamide gel electrophoresis, LTAA has been purified to homogeneity (by 5 independent criteria). The antigen has the following physicochemical properties: molecular weight = 77,000; sub-unit molecular weight = 42,000; Stoke's radius = 40Å; diffusion coefficient = 5.0×10^{7} cm^2 sec^{-1}; sedimentation coefficient = 4.24S and a frictional ratio of 1.42. Efforts are now being made to produce more specific antisera and to develop a radioimmunoassay for the study of circulating LTAA in the sera of lung cancer patients.

Work has been started to modify antibody to CEA so that it is radiopaque and/or radioactive. It could then be monitored by computerized axial tomography or radio-scanning and potentially provide a sensitive method for detecting and localizing tumors smaller than the level of clinical detection by usual means. The model of human adenocarcinoma of colon (GW-39) adapted to transplantation in the hamster will be used initially and these findings then can be transposed to the human system with a minimum of modification.

Efforts are also being directed toward the isolation of surface antigens from mouse virus-induced leukemias. An assay of inhibition of complement-dependent humoral cytotoxicity, using antisera with well-defined specificities, is being used to follow the purification. By use of ion exchange and affinity chromatography, it has been possible to obtain soluble antigens with enriched specific activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05557-09 LID

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cellular Immune Responses in Experimental Tumor Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.B. Herberman	Chief, LID	LID NCI
OTHERS:	H.T. Holden	Microbiologist	LID NCI
	J.R. Oehler	Guest Worker	LID NCI
	M.E. Nunn	Biologist	LID NCI

COOPERATING UNITS (if any)
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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural cell-mediated cytotoxicity of mouse and rat lymphocytes against tumors and other target cells was studied in a short-term ^{51}Cr release assay. Natural killing (NK) in mice was shown to be directed against a variety of mouse tumors, including lymphomas, carcinomas and sarcomas, and against some normal cells: thymus cells, bone marrow cells and macrophages. NK activity against heterologous tumor cells was also demonstrated. The majority of NK cells in mice and rats were found to have Fc receptors for IgG and to bear low densities of T cell markers. NK activity was strongly boosted 3 days after inoculation of antigenic tumor cells, lymphocytic choriomeningitis virus, BCG or C. parvum. This effector mechanism appears to play an important role in in vivo resistance against tumor growth and bone marrow transplantation. The cell-mediated immune response of W/Fu rats to a syngeneic lymphoma was also studied. Upon removal of suppressor macrophages from spleens of immunized rats, lymphoproliferative responses to the tumor cells were shown to be long-lasting. Other parameters of the cell-mediated immune response (secondary generation of cytotoxicity, production of migration inhibitory factor, resistance to tumor challenge, and adoptive transfer of this resistance) were also shown to be persistent.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objectives of this project are: 1) To study natural cell-mediated immunity in rodents and to determine its characteristics and its role in resistance against tumor growth; 2) to induce and measure cell-mediated immune reactions against tumor antigens, and to determine the role of various phases of the immune response in resistance against tumor growth; and 3) to analyze the role of lymphoid cell subpopulations in the immune response, and in the suppression of the immune response to tumor antigens.

Methods Employed: Lymphoid cells are obtained from tumor-bearing, immune, and normal subjects. A variety of normal and tumor cell lines are maintained in tissue culture. Inbred strains of mice and rats, and transplantable tumors induced by viral, chemical or unknown agents are used in model systems. Cellular immune reactions to tumor antigens are measured by mixed lymphocyte tumor cell cultures, migration inhibition, and lymphocyte-mediated cytotoxicity assay. Tumors and other materials are tested for oncogenic and other viruses. Subpopulations of lymphoid cells are prepared by separation of rosette-forming cells, passage through anti-immunoglobulin columns, adherence columns and plates, and by antiserum treatments.

Major Findings:

I. Natural Cell-Mediated Immunity Against Tumor Cells

We have continued to perform extensive studies on natural cell-mediated cytotoxicity against tumor cells and other target cells. During the past year, we have extended some of the findings made in mouse to other species and have found that there are many similarities in natural killer (NK) cells among various species. Our studies have focused on the following areas: a) The specificity of natural cell-mediated cytotoxicity; b) the characteristics of NK cells; c) factors affecting the levels of NK activity; d) relationship of NK activity to in vivo resistance to tumor growth and to bone marrow transplants.

Most of the initial studies with mouse NK cells in a short-term ^{51}Cr release assay were against lymphoma target cells and antigenic specificities associated with endogenous type C viruses. However, our recent studies have indicated a considerably broader range of reactivity. Many mouse carcinomas and sarcomas have been found to be susceptible to NK activity, and there has been some activity against normal thymus cells, bone marrow cells and macrophages. In addition, the cytotoxicity has not been restricted to cells from the same species. Mouse, rat and human NK cells have been found to have considerable reactivity against some, but not all, heterologous target cells. From direct testing and inhibition studies, it appears that NK cells can react against a variety of antigenic specificities.

Detailed studies have been formed on the nature of NK cells. It was initially thought that mouse and rat NK cells were lymphocytes which lacked any identifiable cell surface markers. However, since human NK cells were found to have Fc receptors and low affinity receptors for sheep erythrocytes,

it was of interest to closely reexamine the rodent NK cells for Fc receptors and for T cell markers. Adsorption of mouse and rat NK cells on monolayers of complexes of sheep or mouse erythrocytes and IgG antibodies to the erythrocytes (EA) resulted in a substantial reduction in total cytotoxic activity. In addition, mouse NK cells were shown to form rosettes with EA and to sediment faster in a 1 g velocity sedimentation gradient. In preliminary studies with guinea pig NK cells, similar results have been obtained. These findings indicate that at least the majority of rodent NK cells have receptors for the Fc portion of IgG. To more closely examine the possible T cell derivation of mouse NK cells, repeated studies have been performed with antisera to the Thy 1.2 antigen. Treatment of NK cells from nude mice, or from older conventional mice with NK activity boosted by lymphocytic choriomeningitis virus (LCMV) (see below) resulted in a substantial reduction of NK activity. Repeat treatment of this type caused greater decreases. To confirm that the inhibition effects were really due to Thy 1.2 on the NK cells rather than some other antigens, experiments were performed with specific antisera to Thy 1.2, made in congenic mice by Drs. Shen and Boyse, Sloan Kettering Institute. These antisera had the same effects as our allogeneic AKR anti C3H thymus sera. As a further proof, anti-Thy 1.2 sera were used to treat spleen cells from mice congenic for the Thy 1 trait. Only the NK reactivity of cells from Thy 1.2 mice was reduced. These findings of low amounts of Thy 1.2 on NK cells, plus the previously observed inverse relationship between natural reactivity and thymus function, indicate that NK cells are pre-T cells. Similar to these observations, recent studies with rat NK cells indicate that an anti-T cell serum can inhibit reactivity.

Spontaneous expression of NK reactivity in mice and rats is quite transient, being detectable only at 5-10 weeks of age. However, it has been possible to augment the levels of NK reactivity in young mice, or to cause a reappearance of NK reactivity in older mice, by inoculation of cells bearing the relevant antigens or nonspecifically by inoculation of LCMV, other mouse viruses, BCG or Corynebacterium parvum (C. parvum). All of these treatments produced a peak in cytotoxicity at 3 days, which was detectable in all of the lymphoid organs, except the thymus, and in the bone marrow. Recent studies indicate that C. parvum and some other agents can similarly cause high increases in NK activity in rats. The effector cells mediating the boosted cytotoxicity had the same characteristics as those of NK cells in unstimulated animals.

Studies have also been performed to identify *in vivo* and *in vitro* treatments which can inhibit NK reactivity. Natural reactivity in mice was found to be relatively radioresistant but was inhibited with 850-900 R given 24 hours prior to the test. Treatment of mice with cyclophosphamide or with hydrocortisone caused marked reductions in NK activity, but drugs which inhibit DNA synthesis, methotrexate and cytosine arabinoside, have had no effect on spontaneous levels or on the ability of mice to respond to LCMV augmentation. Treatment of mice with ^{89}Sr, which has been found to inhibit resistance to bone marrow transplants, was found to only have modest inhibitory effects on NK activity. One characteristic of mouse NK activity has been its lability upon *in vitro* incubation at 37°C. This lability, with substantial decreases within a few hours, was found to be quite dependent on the cell density and

culture vessel. It appears likely that different conditions can be found to reliably maintain or abrogate NK activity. Treatment of NK cells with trypsin has been found to reduce or eliminate reactivity. However, when the treated cells were then incubated overnight at 37°C, NK activity was regenerated. These findings, plus the many similarities between mouse NK cells and K cells for antibody-dependent cytotoxicity against mouse tumor target cells, have raised the possibility that natural cytotoxicity is mediated by K cells armed in vivo with natural antibodies, via their Fc receptors. Studies are in progress to carefully investigate this hypothesis.

An increasing amount of data indicate that NK cells may play an important role in in vivo resistance to tumor growth. A number of mouse lymphomas, carcinomas, sarcomas and the B16 melanoma have been found to grow poorly in nude mice, which have high levels of NK activity. Each of these tumors has been shown to be sensitive to NK activity. Some schedules of inoculation of mice with LCMV to boost their NK activity have resulted in increased resistance to tumor growth. In addition to a role in tumor resistance, we have performed studies to determine the possible role of NK cells in resistance to bone marrow transplants. There are a number of analogies between these two phenomena: development at about 3 weeks of age, sensitivity to cyclophosphamide and relative resistance to x-irradiation. In addition, our findings that some bone marrow cells are sensitive to NK activity, that NK activity is detectable in the bone marrow, and that inoculation of allogeneic bone marrow cells can boost NK activity are suggestive of a role of NK in bone marrow resistance.

II. Cellular Immune Reactions in Rats and Factors Affecting These Responses

Rats immune to the Gross leukemia-virus-induced lymphoma, (C58NT)D, have been shown to have lymphoproliferative responses to tumor cells bearing the relevant antigens. However, these responses were only detectable between 15-60 days in rats regressing their tumors and were completely undetectable in rats bearing progressively growing tumors. It has been shown that T lymphocytes with the ability to undergo a proliferative response to (C58NT)D were present at other times in rats inoculated with the tumor, but that suppressor macrophages inhibited the responses. The spleens and peritoneal exudates of even normal rats and immune rats not bearing tumors were found to have suppressor macrophage activity. Removal of these suppressor cells by rayon adherence columns or by treatment with carbonyl iron plus magnet resulted in the ability to demonstrate otherwise undetectable reactivity. The presence of these suppressor cells in normal rat spleens was shown to account for the difficulty in demonstrating their responsiveness in mixed leukocyte cultures. Furthermore, by removal of these suppressor cells, we were able to re-examine the kinetics of the proliferative response of rats inoculated with (C58NT)D. Reactivity was detectable within 10-12 days, reached peak levels between 30-60 days but remained detectable for well over one year. This persistent reactivty was compatible with our previous observations of long-lasting resistance of immunized rats to challenge with tumor cells. Recent studies have indicated that other aspects of the cellular immune response against tumor antigens are also quite persistent. The ability to generate cytoxicity

upon re-exposure to tumor cells in vitro, production of migration inhibitory factor, and systemic adoptive transfer of immunity were all found to be long-lasting. These findings make it more likely that some or all of these responses play a significant role in in vivo resistance against tumor growth.

Significance to Biomedical Research and the Program of the Institute:

Detection of tumor antigens and of immune reactions to tumor-associated antigens is essential for progress in tumor immunology. The sensitive assays of cell-mediated and humoral immunity which have been developed in this laboratory have already proved useful in studying the immune response to animal and human tumors.

The ability of our laboratory to perform multiple assays of cell-mediated immunity to tumor antigens, and to characterize the effector cells, should allow detailed dissection of the role of the immune responses in limiting or preventing tumor growth. These assays may also be useful in immunodiagnosis of cancer and in measuring changes in immunity, in response to chemotherapy or immunotherapy.

Natural cell-mediated immunity may play an important role in immune surveillance against tumors. Understanding of this phenomenon and demonstration of its in vivo significance in mice should be very useful for the understanding of the significance of human natural cell-mediated immunity, which seems to be mediated by the same or very similar types of effector cells.

Proposed Course of Project:

Studies on natural cell-mediated cytotoxicity in mice and rats are being continued. A major emphasis will be on a detailed evaluation of the role of NK cells in in vivo resistance against tumor growth and against bone marrow transplants. Mice will be treated in a variety of ways to increase or decrease their levels of NK activity, and the effects of the treatments on resistance will be examined. The other main focus of our attention will be on the relationship between NK cells and antibody-dependent cell-mediated cytotoxicity. A series of in vitro studies will be performed to determine whether natural antibodies can arm K cells and thereby duplicate the pattern of NK activity, and whether B cells are needed for in vitro regeneration of NK activity.

Studies on cellular immunity of rats to the (C58NT)D lymphoma will be continued. Attention will be directed toward a variant of (C58NT)D, which grows progressively in adult rats despite its induction of a vigorous T cell cytotoxic response. Attempts will be made to determine the mechanism for the escape of this tumor from the immune response of the host. This should provide considerable insight into the important mechanisms for in vivo resistance to growth of this tumor.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08511-02 LID

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Characterization and Measurement of Soluble Antigens Associated with Neoplastic Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.00	4.00	1.00

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Circulating tumor markers are being studied to aid in detection and diagnosis and monitoring the course of patients with various neoplastic diseases. Sensitive quantitation of alpha-fetoprotein (AFP) by radioimmunoassay has been shown to be of considerable value for following therapy in patients with hepatocellular carcinoma (HC) and testicular cancer of germ cell origin where quantitation of human chorionic gonadotropin (HCG) also serves as an indicator of tumor activity. Experimental studies in primates with chemically induced HC allow the demonstration of the usefulness of AFP for early detection of tumor and also to calculate the synthetic rate and catabolic rate of AFP to estimate the total body tumor load. Similar studies are being started with patients having testicular germ cell tumors. A new human lung tumor-associated antigen (LTAA) has been isolated and characterized. The labeled antigen is being used to set up a sensitive RIA for the quantitation of LTAA levels in sera of patients with lung cancer and other disease conditions. Similar studies are being performed using a murine virus-induced leukemia (MBL-2) as a model system and quantitating tumor-associated antigen(s) by an antibody- and complement-mediated cytotoxicity (^{51}Cr release) assay.

Project Description:

I. Characterization and Measurement of Soluble Antigens Associated with Human Neoplasms. Objective 6, Approach 1

A. Studies of Alpha-Fetoprotein (AFP)

1. Quantitation of AFP in human disease: Serum AFP assays are performed on selected groups of patients where it is known to be an effective marker for tumor activity. Hepatoma patients from the NIH Clinical Center, Washington V.A. Hospital, the Mayo Clinic, and Lusaka, Zambia, are being followed for demonstration of the effectiveness of chemotherapy. Several patients have been noted to have complete or partial remission on adriamycin therapy (manuscripts submitted). The combined use of AFP with human chorionic gonadotropin has been demonstrated to provide an accurate tumor marker for about 90% of patients with non-seminomatous testicular germ cell tumors. One-fourth of the patients have marker levels (one or both) fall to normal after successful therapy or continue to rise with no response to therapy, and about one-third of the patients show a transient fall in markers with partial response to therapy but then a rise which preceded by 2-14 months the clinical recurrence of tumor. In almost one-half of the patients the markers both fall in response to therapy but only one of the markers increases in association with recurrent tumor. This demonstrates that the markers are produced by different cell types which may respond differently to therapy and emphasizes the advantage of using both markers simultaneously. The two markers provide evidence of sub-clinical tumor that can reduce the error in staging by clinical means alone. In several collaborative studies utilizing combined modalities of therapy the regular quantitation of AFP and HCG has become an extremely important aspect of the evaluation of therapy. Consecutive rising values of either invariably have meant that the tumor has become resistant to the present therapy or somehow has started to grow and will be clinically evident within a few months -- it signals the time to consider a change in therapy.

During the past year the assay for AFP has been improved for greater sensitivity. The mean normal adult level is now 3.5 ng/ml and the assay sensitivity is 500 pgm/ml (formerly 20 ng/ml and 5.5 ng/ml, respectively). We are in the process of obtaining normal values to look for variation with sex, age and race and also to re-evaluate the low (? normal) levels seen in patients with hepatocellular carcinoma, germ cell tumors and other conditions where minor elevations might be expected.

2. Study of AFP in primates: Studies on the early detection of hepatocellular carcinoma in monkeys treated with chemical carcinogens are continuing. The AFP serum level is clearly elevated many months before the tumor is clinically detectable and increases with increasing tumor size. During the past year we have developed a sensitive radioimmunoassay (RIA) for monkey AFP to replace the previous modification of the human AFP assay. Experimental chemotherapeutic agents are now being tried in monkeys with hepatocellular carcinoma and the results are monitored by serum AFP levels.

3. Metabolism of AFP: In vitro synthesis of AFP in 5 different carcinogen-induced monkey hepatomas was demonstrated by both specific incorporation of ^{14}C amino acids and AFP quantitation in tissue culture media in 4 of the tumors. Synthetic rates were 0.37-5.5 ng AFP/mg tumor/day and different tumor nodules from the same monkey had very similar synthetic rates. The degree of tumor cell differentiation showed an inverse correlation with the synthetic rate of AFP.

Extensive studies of AFP turnover in monkeys demonstrated that labeled (^{125}I) AFP, by three methods, was damaged or biologically changed so that from 30 to 60% of the AFP was rapidly degraded in the first 2 days after injection when compared to unlabeled, "native" AFP. The damaged AFP could be removed by preparative electrophoresis or by biological "screening" and the undamaged labeled AFP then had a plasma disappearance curve very similar to unlabeled AFP. Further experiments will compare the turnover of AFP in monkeys with hepatomas and varying levels of plasma AFP.

4. AFP in combination with other markers: Serum AFP and HCG levels are being compared with levels of carcinoembryonic antigen (CEA), alkaline phosphatase and other potential tumor markers in various gastrointestinal malignancies to obtain evidence of correlation and/or discordance with tumor size. Serial serums will be obtained from patients having objective changes in tumor size, either increase or decrease.

The in vitro synthesis of both AFP and HCG will be studied in biopsy specimens from patients with testicular germ cell tumors producing one or both markers. These patients are entered in a study designed to ascertain the benefit of surgical removal of a significant portion of tumor in addition to chemotherapy. The synthetic rates along with known turnover rates and the total circulating markers will be used to estimate the total body tumor burden.

5. Immunologic staining of tumor for markers: Specific antiserum to AFP and HCG has been used together with a second antibody conjugated to horseradish peroxidase to identify the cells associated with each of these markers. HCG is identified with syncytiotrophoblastic giant cells and choriocarcinoma while AFP is found in embryonal carcinoma cells and endodermal sinus tumor. The tissues were formalin fixed and adjacent sections showed no evidence of any cells staining for both markers. The stain for HCG was as sensitive as the serum level in that all patients with serum elevation of HCG were found to have positively stained syncytiotrophoblasts or choriocarcinoma. The stain for AFP was consistent in demonstrating positive cells only in patients with serum levels greater than 500 ng/ml; in those with serum levels from 500 ng/ml down to normal levels, some had positive cells in tumor sections but others did not. This demonstrates the ability to identify markers retrospectively, at a time when serum may be unavailable.

B. Studies of Lung Tumor-Associated Antigens (LTAA). Objective 5, Approach 4

1. Isolation of LTAA: Work has continued on the isolation of an antigen from a saline extract of a lung adenocarcinoma (LT-118). Monitoring of the isolation has been carried out using rabbit antisera (R-152) raised against the pooled extracts of 6 different lung tumors and absorbed with extracts of normal lungs, normal human plasma and A, B, O erythrocytes. The absorbed antiserum had activity against extracts of lung tumors, but not with extracts of normal lungs or other normal tissues. Antiserum, R-152, was used to isolate the antigen by affinity chromatography. When analyzed by acrylamide gel electrophoresis, the material which was eluted from the antibody coupled to Sepharose contained proteins of 5 different mobilities. This mixture was labeled with ^{125}I and the protein with greatest immunoreactivity with R-152 was identified by two-dimensional immunoelectrophoresis. This single protein was then purified by preparative acrylamide gel electrophoresis (PAGE). This protein was judged homogeneous by 5 independent criteria: 1) PAGE, 2) SDS-PAGE, 3) DEAE chromatography, 4) sieving chromatography (G-200), and 5) gradient ultracentrifugation. The antigen has the following physicochemical properties: molecular weight = 77,000; sub-unit molecular weight = 42,000; Stoke's radius = 40Å; diffusion coefficient = 5.0×10^{-7} cm^2 sec^{-1}; sedimentation coefficient = 4.24S and a frictional ratio of 1.42. Preliminary experiments indicate the presence of carbohydrate.

We have examined the extract of another human lung tumor (LT-109) for the presence of this same LTAA. By adding purified radioiodinated antigen from LT-118 to the crude saline extract of LT-109 we have isolated a homogeneous protein by ion exchange chromatography, molecular sieve chromatography and preparative PAGE. This protein is immunologically identical to the LTAA from LT-118 and appears to be very similar in physicochemical characteristics.

2. Development of antisera: A second rabbit (R-201) antiserum has been developed using aliquots of the same antigenic pool used to immunize R-152. This antiserum has been absorbed using only insolubilized immunosorbants, and appears suitable for use in a radioimmunoassay. Attempts are in progress to develop better antisera in monkeys and goats against antigen isolated as above.

3. Quantitative assays: Radial diffusion into antibody-containing agar gel was developed as a method of quantitation; the sensitivity being approximately 20 times greater than double diffusion. This assay was still not sufficiently sensitive to detect LTAA in serum and was of limited usefulness in monitoring isolation procedures since the threshold was 1-5 µg/ml.

Preliminary work is now under way to develop a double antibody RIA with sensitivity in the nanogram range. Several clinical collaborations have been set up to collect serial serums from patients with lung cancer who are being treated by surgery and chemotherapy to determine the circulating

levels of LTAA and the correlation of this LTAA with changes in tumor size. The LTAA is also being tested in lung cancer patients for delayed hypersensitivity skin reactivity and in vitro against leukocytes from patients with lung cancer in the leukocyte migration inhibition assay. These studies are in progress and too early to evaluate.

4. Immunolocalization of Tumors: Work has been started to modify antibody to CEA so that it is radiopaque and/or radioactive. It could then be monitored by computerized axial tomography or radio-scanning and potentially provide a sensitive method for detecting and localizing tumors smaller than the level of clinical detection by usual means. The model of human adenocarcinoma of colon (GW-39) adapted to transplantation in the hamster will be used initially and these findings then can be transposed to the human system with a minimum of modification.

II. Tumor-Associated Antigens in Mice. Objective 3, Approach 5

A. Demonstration of Surface Antigen(s) from a Moloney Leukemia Virus-induced Tumor (MBL-2)

1. Isolation of MBL-2 antigens: Attempts have been made to purify the antigens identified by antisera in an antibody-mediated cytotoxicity assay. Both the 3M KCl extract of tumor cells and the ascites supernatant have provided starting material and have been fractionated by four different procedures: ammonium sulfate salting out, ion exchange chromatography, antibody affinity chromatography and ultracentrifugation in potassium bromide gradient. Each of these methods has provided enriched antigen activity, but it appears obvious that multiple-step isolation procedures will be necessary to achieve homogeneity of the antigen activity(s). Other methods are now being tried.

2. Evaluation of MBL-2 antigens: Analysis of the relationship of the tumor-associated antigens of MBL-2 to type C viral proteins has been carried out by the direct reaction of MBL-2 cells with antisera to various murine leukemia viruses and antisera to purified viral peptides and glycopeptides. No specific complement-mediated cytolysis was observed.

The MBL-2 cells have been surface labeled with ^{125}I by the lactoperoxidase method and then dissociated by detergent (NP 40). The soluble surface antigens are then reacted with antisera made in rabbits against MBL-2 or in mice by injecting MSV. The immune precipitate is then solubilized in detergent and separated on SDS-PAGE.

3. Specificity of antisera to MBL-2: Rabbit anti-MBL-2 serum and antisera from mice injected with murine sarcoma virus were both tested for inhibition of cytotoxicity with a variety of tumor extracts and viral preparations. Both antisera were inhibited by whole cells of the following tumors: MBL-2, RBL-5, FBL-3, EL-4, LSTRA, MCDV-12 and YC8. They were also inhibited by MLV, RLV, p30 from RLV and the 3M KCl extracts of RBL-5 and EL-4 tumors as well as MBL-2. The murine mammary tumor virus and

3M KCl extracts of normal spleen cells and E♂G2 and FBL-3 tumor cells were not active in the inhibition assay. Both antisera appeared to show similar specificities against Moloney and Rauscher viral antigens.

B. AFP in Mice

1. Quantitation: Specific antisera to mouse AFP has been developed and has been used in an electroimmunodiffusion assay to quantitate down to the level of 1 µg/ml. We are currently isolating mouse AFP for a RIA which should be sensitive to a few nanograms.

2. Association with tumors: AFP synthesis by tumor was originally reported for transplantable mouse hepatomas. We now have several transplantable spontaneous hepatomas in the C3Hf/A^{vy} strain which produce AFP which can serve as a model for a variety of conditions and therapeutic manipulations. Once the RIA measurement of AFP is available other tumors, both spontaneous and induced, can also be added to the model system.

Significance for Biomedical Research:

There are now many circulating substances which can be assayed in the blood of individuals with neoplastic diseases which are reported to be associated with the tumor process. Some of these substances are obviously more specific for malignancy than others. Those substances that are the most specific have the potential for being utilized as aids to early diagnosis (possibly for screening) and for localization of tumor, but have the immediate advantage in serving as tumor markers to monitor the effectiveness of therapy. All visceral solid tumors have a common disadvantage in not being easily measured or otherwise evaluated for progression or regression. This laboratory is committed to the clinical application of known tumor markers (AFP, HCG and CEA) as well as to the discovery of new ones (LTAA). In addition to demonstrating an association between certain tumors and markers, we are interested in studying the metabolism of the markers to further define the precise relationship. It is possible that the tumor markers or antisera directed against them could have some value in immunotherapy or even in prevention. This research on tumor markers falls under Objective VI, Approaches 1 and 2 and Objective V, Approaches 2, 3 and 4 of the National Cancer Plan.

The study of cell surface antigens and ways to detect and quantitate them will provide basic information needed to understand the process of carcinogenesis and malignant transformation. Animal models and in vitro systems are frequently used because of the ease of manipulation and control of variables. We are searching for membrane antigens since those on the surface of the cell are the most likely to trigger immune recognition and also serve as targets for immunologically directed therapy. This research falls under Objective III, Approaches 4 and 5.

Proposed Course of Project:

This project represents our laboratory's continued commitment to immunodiagnosis utilizing circulating antigens (released or secreted by tumor cells) and cell surface antigens. We will continue to derive information as to their usefulness in following the course of malignant disease and how they may be used in combination to aid the early diagnosis of cancer.

We will continue to use AFP, HCG and CEA measured by sensitive RIA to monitor patients with hepatocellular carcinoma, pancreatic carcinoma, and testicular and ovarian carcinoma. We will use the carcinogen-induced hepatomas in primates to study the relationship of AFP to tumor burden by measuring synthetic rates and catabolic rates of AFP in the animals with tumor. We will begin similar metabolic studies in patients with testicular germ cell tumors utilizing HCG in addition to AFP. We will continue to perform tissue staining for AFP and HCG as an aid to histologic classification of testicular tumors.

We will begin quantitating LTAA in serum of various disease conditions in addition to patients with lung cancer to determine the correlation with tumor and the potential for aiding early diagnosis.

We will be working on labeling techniques to modify antibody to tumor-associated antigens to enable the localization of small tumor nodules (subclinical) by computerized axial tomography or radio-scanning.

We will continue to search for tumor-associated antigens in mouse tumors to extend our basic knowledge of the process of transformation and of the host response to tumor. The techniques applied in the animal model system are transferable with minor modifications to the investigation of human malignancy.

Since several human tumors have been shown to have antigenic activity in assays of cell-mediated immunity (CMI), we expect to choose one of these for development of antisera to the protein or glycoprotein responsible for the CMI. After specific antiserum is developed this can be used to monitor further isolation of the antigen and development of a RIA for the sensitive determination of antigen levels in serum.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08500-03 LID

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Immunodiagnosis, Immunotherapy and Immunological Monitoring in Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.D. Bonnard	Visiting Scientist	LID	NCI	
OTHERS:	R. B. Herberman	Chief	LID	NCI	
	A. Levin	Guest Worker	LID	NCI	
	W. H. West	Research Associate	LID	NCI	
	R. Richardson	Clinical Associate	LID	NCI	
	J. R. Ortaldo	Biologist	LID	NCI	
	H. D. Kay	IPA	LID	NCI	
	J. L. Weese	Guest Worker	LID	NCI	(until 6/30/77)
	A. Morales	Guest Worker	LID	NCI	(until 10/15/76)
	A. Kibrite	Guest Worker	LID	NCI	(until 11/30/76)
	V. Tinley	IPA	LID	NCI	(from 10/13/76)
	R. Maca	IPA	LID	NCI	(from 7/1/77)
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COOPERATING UNITS (if any) Litton-Bionetics, Inc.; G.W. Univ.; R,NCI; M,NCI; Surg,NCI; PO,NCI; LTCB,NCI; Met,NCI; U. of Mich.; Kingston Univ.; Laval Univ.; Georgetown Univ.; Scripps Clin.; Natl. Naval Med. Ctr.; Walter Reed Med. Ctr.; Portsmouth Naval Hosp.; Univ. of S.C.

LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.50	6.80	1.70

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Characterization of natural cytotoxicity and natural killer cells. Comparison with lymphocytes that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against Chang liver cells. Study of persistence of natural cytotoxicity and ADCC in 5-7 day cultures. Study of these activities in mixed lymphocyte cultures. Enhancement of these activities by co-cultures with various antigens, mitogens and with tumor cells. Comparison of these activities with the generation of specific immune T lymphocytes. Use of continuous cultures of pure T cells from any given individual; characterization of their cell surface markers and functional activities. Immunological monitoring of patients with lung and breast cancer, Ewing's sarcoma, and melanoma. Adjuvant chemotherapy and immunotherapy with BCG, C. parvum, with or without irradiated allogeneic tumor cells. Skin tests with highly purified soluble extracts of tumor cells. In vitro assessment of immunocompetence, natural cytotoxicity, and tumor immune reactivity, by short-term cytotoxicity assays, mixed lymphocyte tumor cell culture, and leukocyte migration inhibition. Reactivity of subpopulation of lymphocytes. Examination of cells from various lymphoid organs and from the tumor.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

I. Studies on Cytotoxicity

We initiated a thorough investigation on the specificity of diverse cytotoxic reactions in vitro, and on the effector cells involved. We define direct cytotoxicity as that measured in a 4-hr. ^{51}Cr release assay (CRA) with fresh mononuclear peripheral blood leukocytes (MPBL) or their subpopulations. We tried to characterize the effector cells of the spontaneous cytotoxicity or natural killing (NK), and to identify specifically immune cytotoxic lymphocytes (CTL), with reactivity against tumor-associated antigens (TAA). We examined the possible role of natural antibodies and antibody-dependent cell-mediated cytotoxicity (ADCC) in NK. We performed cell separations on the basis of cell-surface markers or size, and used the purified effector cells. More generally, we improved the logistics and the value of direct cytotoxicity experiments.

We started a similar investigation on the specificity of indirect cytotoxic reactions generated in vitro, and on the precursor cells and effector cells involved. We define indirect cytotoxicity as that assessed at the end of a culture period of 5-7 days, with MPBL cultured alone, or with various stimuli, including tumor cells. We compared the mechanisms of the indirect cytotoxicity with those of well-defined cytotoxic reactions, such as NK, ADCC, and the cell-mediated lympholysis (CML) assay. Efforts were made to ascertain the possible role of antibodies and ADCC reactions. Cell separations were done at the onset and at the end of cultures, and the various fractions tested in the assays. More generally, the logistics of indirect cytotoxicity experiments was improved.

We tried to develop new procedures for the study of the specifity of cytotoxic reactions in humans. We made use of the cold target cell competitive inhibition test for analyzing the specificity of NK. We also tried selective adsorption on target-cell monolayers of NK and immune CTL activities. Finally, we devised useful ways to analyze the cytotoxicity data on several target cells with regard to specificity.

II. Cell Separation Studies and Generation of Pure T Cells

The usefulness of cytotoxicity experiments, and other assays of cell-mediated immunity, as correlates for in vivo tumor immunity and rejection, depends in our opinion largely on the precise identification of the cells involved in each step of the reactions. Therefore, we have utilized a very systematic approach with separation of MPBL into various subpopulations, on the basis of cell surface markers and differences in size, with close monitoring of the resulting fractions for morphology, cell-surface markers, and subsequent testing of these fractions in several functional assays.

We initiated a new series of studies when we became aware of the possibility of continuously growing T cells from any given individual, which appeared to retain the functional capacities of fresh T cells. Our major objectives with these cultivated T cells was to determine whether or not they have immunological memory and whether they could display the whole spectrum of T cell functions and characteristics. We intend to collaborate on the development of the system in an animal model, and to study the possibility of performing immunological reconstitution and immunotherapy with these cells. We also intend to carefully examine the functions and usefulness of cultured T cells from cancer patients.

III. Immune Monitoring of Cancer Patients

A major effort of the Laboratory of Immunodiagnosis has been the immunological monitoring of cancer patients with in vivo and in vitro tests. Protocols have been developed with collaborating institutions, where patients receive primary care, for this purpose. The emphasis has been on patients with carcinomas of the lung and breast, and on melanoma and Ewing's sarcoma. The overall goals of this project are: (1) to detect tumor-specific and tumor-associated antigens (TAA) on tumor cells; (2) to detect cellular and humoral immune reactions to tumor antigens; (3) to evaluate the diagnostic, prognostic and therapeutic significance of cellular immune responses to TAA; (4) to develop and evaluate assays for the immunological monitoring and immunodiagnosis of human cancer; (5) to isolate and characterize TAA and other cell surface antigens; (6) to test the reagents developed under (5) in certain in vitro assays as well as in delayed hypersensitivity reactions in these tumor systems; (7) to monitor general immunological competence in cancer bearers; (8) to evaluate the effects on the immune response of inhibitory phenomena associated with tumor cells and tumor cell growth. The aims of this part of the Laboratory were: (1) to design protocols for the immunological monitoring of cancer patients at the collaborating institutions; (2) to organize the logistics regarding these protocols, including regular patient follow-up, collection of blood samples and tumor specimens, and distribution into the proper tests in the testing laboratory; (3) to perform skin tests with recall antigens and purified tumor extracts, and to help in setting priorities for the step by step purification of tumor-derived antigens; and (4) to help evaluate and summarize for publication the serial clinical data, and the immunological data obtained in the testing laboratory.

Major Findings:

I. Natural Reactivity and Tumor Immunity in Cytotoxicity Assays

A. The Natural Killer (NK) Cell.

There have been several problems with the clinical use of cytotoxicity assays. In particular, many groups have now recognized the major problems of spontaneous cytotoxicity by normal MPBL from healthy donors, or natural killing (NK). This tends to blur other types of reactivity, which may be present in patients against selected target cells. Therefore, we have been performing

intensive studies on the nature of NK, and on the characteristics of NK cells. We have sought to get information that would allow discrimination and separation of NK from other types of cytotoxicity, particularly disease-related immune reactivity of CTL. It was important to measure NK simultaneously with ADCC, a well-known functional activity of an FcR-positive population of lymphocytes, and whenever possible, with the activity of alloimmune CTL.

The cytotoxicity tests were performed in a 4-hr CRA with fresh, normal MPBL against the K-562 myeloid cell line in the case of NK activity, with fresh MPBL against rabbit antiserum sensitized Chang liver cells in the case of ADCC and with mixed lymphocyte culture (MLC) sensitized alloimmune lymphocytes against PHA-blasts in the case of CML.

NK cells were first characterized as lymphocytes having an Fc receptor (FcR), and in contrast to previous reports, no complement receptor (C3R). The NK cells also demonstrated low-affinity binding to sheep erythrocytes (sE), and therefore appeared to belong to a subpopulation of T cells.

K cells active in ADCC against Chang target cells represent a well-characterized FcR-positive lymphocyte population. We showed that NK cells and K cells could not be physically separated from each other by several different techniques, and raised the possibility that they were within the same lymphocyte subpopulation. In particular, the sE-rosetting procedures used revealed that the majority of K cells were also low-affinity binding sE-RFC (rosette-forming cells).

These results on the nature of the NK and K cells were somewhat at variance with reports from other laboratories: (1) We were successful in detecting NK and K cell activity in sE-RFC pellets, because we avoided NH_4Cl solution lysis of the sE, a procedure which inhibits NK and ADCC. (2) We used sE-rosetting procedures that were demonstrably more effective than those used in most other laboratories. (3) We calculated results in lytic units per fraction, rather than in percent cytotoxicity, which revealed the greater amount of lytic activity recovered in the sE-RFC pellet, as compared with the interface. (4) We used very pure 19S Ig antibodies to perform the EAC-RFC assay, and demonstrated that minimal contamination by 7S IgG would allow this reagent to effectively bind to FcR-positive NK and K cells.

Although they remained physically inseparable, there were noticeable differences between the NK and K cells and/or their mechanisms of action. Trypsin treatment of the effector cells abolished NK, and left ADCC intact; also, Protein A abolished ADCC, and left NK intact. These data were consistent with the hypothesis that NK cells could simply be K cells that were "armed" in vivo with natural antibodies against K-562, and various other cell lines.

Additional evidence for similarities between NK and K cells was obtained from experiments in which the MPBL were cultivated, either alone or in MLC,

and the indirect cytotoxicity was tested in CRA against NK, ADCC and CML target cells as defined above. It became quite clear that cytotoxic activities with specificity for the NK and ADCC targets, were maintained in cultures of MPBL alone, and were also present in MLC reactions. This was called cytotoxicity of cultured cells (CCC) with features of NK and ADCC. The source of serum was quite important, with CCC developing in fetal bovine serum (FBS) much better than in (HuS). The antigenic requirements for the induction of CCC have not yet been examined. Of interest was our observation that high levels of CCC were also obtained, and this even in HuS, when various mitogens and various preparation of mumps virus were used as stimulating agents. The data also indicated that CCC was mediated by FcR-positive effector cells, whereas MLC-CML was mediated by FcR-negative CTL. The culture systems have provided an opportunity for detailed analysis of the kinetics (appearance, disappearance) of the sE receptor and FcR during culture, and this is now being performed.

Overall, the above data allowed us to make a clear distinction between the NK cells and immune CTL, opening avenues of research aimed at identifying both types of effector cells against tumor-associated antigens. The distinction between NK cells and K cells was much less clear, and the possibility remained that NK cells and K cells were the same, with NK representing a K cell "armed" in vivo.

B. Specificity of NK Activity

The broad cytotoxicity mediated by fresh MPBL of healthy donors and patients alike against a variety of tumor-derived target cell-lines has often been called nonspecific. We have analyzed this by the competitive inhibition assay, using unlabeled target cells as inhibitors of CRA. We found that the NK activity defined in the K-562 system represented the activity of cells with a broad but demonstrable specificity. A majority of tumor cell lines used as inhibitors of the CRA against K-562 target cells were effective. Among them, one could distinguish one breast cancer-derived cell line, G-11, that inhibited particularly well. This line was also susceptible to NK, when used as target. Fresh tumor cells (lung, breast) were also inhibitory. Noticeably, a few B cell lines, ALL and CML blasts, and fresh MPBL or PHA-blasts failed completely to inhibit.

C. Attempts to Discriminate NK and Disease-Related Cytotoxicity

It was possible to use what we had recently learned about the NK cells and their specificity in attempts to discriminate this NK activity from disease-related cytotoxicity.

First, we compared the levels of cytotoxicity for several groups of donors against K-562, G-11 and MCF-7, a well-characterized breast cancer cell line. Paired comparisons were made on two-dimensional graphs and donors with increased cytotoxicity against G-11 and MCF-7 relative to NK against K-562 were singled out. A significantly higher frequency of increased reactivity against G-11 was observed with patients with breast cancer but also

with normal male donors and patients with benign breast disease. A more breast cancer related pattern of increased reactivity was obtained with the MCF-7 cell line. Patients with other cancers also had more often relatively increased reactivity against G-11, but not so against MCF-7. The study singled out MCF-7 as a good target for reactivity of breast cancer patients with little susceptibility to NK, whereas G-11 was more susceptible to NK. Similar studies, including those on cell lines derived from tumors of other histologic types, are obviously needed to see whether patients with breast cancer have specific reactivity against MCF-7.

In a further effort to dissect out the cytotoxic effector cells in MPBL of normals and patients, use was made of the presence of FcR on NK cells active against K-562. Immunoadsorption experiments were carried out on sEA monolayers, and cytotoxicity was then tested using the nonadherent fraction of lymphocytes. This procedure almost completely eliminated cytotoxicity against K-562 and G-11, and also diminished the cytotoxicity against lung or melanoma lines. However, a few individuals were identified whose MPBL obviously had an FcR-negative population that was responsible for cytotoxicity against the cell lines other than K-562. At the present time, it is unclear whether these effector cells represent immune CTL.

Immunoadsorption studies were also performed on G-11 monolayers, and on monolayers prepared with other cell lines. Further work is needed to establish whether significant levels of activity against K-562 could then be reproducibly adsorbed, and whether adsorption on one line removed effector cells against the other lines. At least, these tests seemed technically feasible. This contrasts with the difficulties we have experienced when trying to attach K-562 to a flat surface by a variety of techniques.

II. Cell Separation Studies and Generation of Pure T Cells

A. Cell Separations and Monitoring of Cell Surface Markers

1. Requirement for monocytes for functional activities: By the passage of MPBL over Sephadex G-10 columns it was possible to reduce the monocytes (defined by external staining) to a minimum, i.e., to concentrations below 0.1%. This was accompanied by a substantial decrease in the proliferative responses of lymphocytes to the mitogen Con A. Recovery of lymphocytes was often as high as 90% of the input. Also, we found that separation of sE-RFC from MPBL by sedimentation on Ficoll-Hypaque resulted in sE-RFC that were almost completely devoid of monocytes, and this abolished the proliferative responses of these sE-RFC pellets to Con A. Adequate responses were obtained by addition of small numbers of non-E-RFC, which contained a high concentration of monocytes.

2. Functions associated with sE-RFC vs. non-sE-RFC: We continued to use sE rosetting techniques and flotation on Ficoll-Hypaque for the purification of T cells in the sE-RFC pellet. A protocol with two centrifugation steps resulted in excellent purity of the sE-RFC, and in a non-sE-RFC interface population. Under these circumstances, and given adequate numbers of monocytes, the sE-RFC had proliferative responses to PHA, Con A, and antigens

like PPD and mumps, whereas cells from the interface had no such responses. Protocols were designed for the application of these techniques to tests of cell-mediated tumor immunity, namely to the mixed lymphocyte tumor interaction (MLTI), the production of leukocyte migration inhibition factor (LIF), or cytotoxicity experiments performed with patients' MPBL.

3. Subpopulations of T cells: Distinct subpopulations of T cells were identified on the basis of their affinity for sE. When the sE-RFC assay was done at 8°C, with an optimal ratio of sE to MPBL, the total number of sE-RFC was identified (77 ± 4%). However, the same technique performed at 29°C, with a lower ratio of sE to MPBL, identified a subpopulation of high-affinity sE-RFC (54 ± 5%). A "low-affinity" subset was defined by the arithmetical difference between these percentages, as cells which form rosettes only at 8°C but not at 29°C. These low-affinity sE-RFC (23 ± 3%) could be sedimented through Ficoll-Hypaque gradients after rerosetting at 8°C the cells which had not sedimented during an earlier sE separation done at 29°C. These low-affinity sE-RFC have an FcR, and they were shown to represent the vast majority of FcR-positive lymphocytes in MPBL. They were similarly prominent in spleen cells, but not in lymph nodes; they were absent from the thymus, as assessed both by the sE-RFC characteristics and the presence of an FcR. There was an excellent correlation between the presence of these FcR-positive, low-affinity sE-binding cells in lymphocyte preparations, and the ability of such populations to mediate NK or ADCC. Thus, lymph node cells and thymus cells had little or no activity, with regard to NK and ADCC. Individuals with lower numbers of FcR-positive or low-affinity sE-RFC were less active in NK and ADCC assays. In contrast, evidence in this and other laboratories has suggested that precursors and effector cells for MLC-CML reactions were in the high-affinity, FcR-negative subpopulation of T cells.

4. Separation of the cellular components of human tumors: Almost all studies of cell-mediated immunity in cancer patients have been performed with MPBL. However, cells within tumors themselves may be more relevant to responses to tumors, particularly in regard to resistance against tumor growth. Therefore, we initiated a study to separate and examine the various cellular components of human tumors. We used two techniques for separation of cells on the basis of size: 1 g velocity sedimentation and isokinetic fractionation. These methods were worked out with MPBL first, and the same separation procedures are now being used on tumor specimens, particularly lung cancers.

B. Generation and Continuous Growth of Pure T Cells

During the period under review, we had an opportunity to collaborate with Drs. D. Morgan, F. Ruscetti and R. Gallo (Litton-Bionetics, Inc., and Laboratory of Tumor Cell Biology, NCI) on a study of cultured T cells. These cultures could be initiated with MPBL from most normal donors with the action of conditioned media from allogeneic PHA-stimulated lymphocytes. The presence of 20% of the conditioned media (CM) was a prerequisite for the growth of the T cells, at all passage levels. When taken off the conditioned

medium, cells remained viable only for a few days, and stopped proliferating. These T cells were not transformed, did not carry the Epstein-Barr virus nuclear antigen and had a normal karyotype. One of these cultures was 11 months old. Similar T cell cultures could be initiated from human bone marrows and spleens.

Our studies have shown the cultures to be virtually pure T cells, with no evidence for B cells. These cells were >98% sE-RFC, with 70% showing high-affinity binding when grown on the conditioned media. However, when taken off the conditioned media, >90% of the cells demonstrated high-affinity binding to sE-RFC. Only a small proportion (0-4%) had detectable FcR, as measured by the technique of rosetting with chicken-erythrocyte-antibody complexes, and this only when the cells were off conditioned media. Also very few cells had receptors for complement (0-4%), as measured by rosetting with sEAC complexes. There was no immunofluorescent staining with a goat anti-IgM reagent, and the cells did not form rosettes with mouse erythrocytes.

A growth promoting factor was found in the CM that was separable from contaminating PHA, and this is now being characterized. The cultured T cells displayed proliferative responses to alloantigens in MLC, but their responses to the T cell mitogens PHA, Con A and PWM were lost after a few passages. Preliminary evidence suggested that this was because of lack of adequate numbers of monocytes in these cultures. The role of monocytes in response to T cell mitogens is now being examined in this model. Similarly, no responses were obtained against the antigens PPD and mumps in the absence of added macrophages. The T cells after 5 days in culture never responded to B cell mitogens, with or without macrophages added. Considerably more work is needed to assess the potential of using such cultured T cells, rather than MPBL for various in vitro assays, and for immunotherapy.

III. Immune Monitoring of Cancer Patients

A. In Vivo Delayed Hypersensitivity Testing of Tumor Immunity

We continued to measure delayed hypersensitivity reactions of patients to Sephadex G-200 and polyacrylamide gel electrophoresis (PAGE) purified soluble fractions of tumor cell extracts, prepared by Dr. A. Hollinshead. The major emphasis during the past year has been to test large numbers of cancer patients, with large batches of purified extracts prepared from standardized sources, mainly tissue culture cell lines shown to be antigenic in in vitro assays. The main concern has been to verify, in this large-scale testing, the specificity of these skin test antigens, which had been suggested by earlier studies with small amounts of materials and small numbers of patients.

Some PAGE fractions from the MCF-7 tissue culture line have been shown to give positive skin tests in many breast cancer patients (16/38), and only in a small proportion of patients with other types of cancer. Therefore, we have been able to confirm the breast cancer-associated specificity of this

material, and we are currently using it for serial tests of all patients on our clinical breast cancer protocol to determine the relationship of reactivity to clinical status and response to therapy.

A Sephadex melanoma preparation derived from one large metastatic lesion to the liver was not sufficiently specific for melanoma. We are therefore further purifying the preparation by PAGE. We also are evaluating the antigenicity of some melanoma-derived tissue culture lines, and other melanoma tumor preparations.

Extracts have been prepared from the lung cancer-derived cell line CaLu-1, and from the Ewing's sarcoma-derived cell line 5838, both of which were selected because of evidence of specific antigens in the leukocyte migration inhibition (LMI) test. Each preparation was found to give positive skin test reactions; further separation and testing for specificity are in progress.

B. 29°C and 8°C Rosette Assays

We further emphasized the enumeration of the percent of T cells in the peripheral blood by rosette formation with sheep erythrocytes. The test was performed under suboptimal conditions, at 29°C, as well as under optimal conditions at 8°C. The technique has been very well standardized, and this allowed considerable testing of cancer patients, including more longitudinal follow-up studies. In our study of serial monitoring of post-operative lung cancer patients, the rosette results have shown much promise in their clinical correlations, and particularly in their ability to predict recurrence of disease. Thus 26/36 patients with recurring disease had decreased "high-affinity" sE-RFC values a mean of 3 months before clinical signs of recurrence. This contrasts with decreased high-affinity E-RFC in only 2/20 patients without evidence of further disease. With breast cancer patients we found that those on adjuvant chemotherapy had considerably depressed values of "high-affinity" sE-RFC, but there was a strong trend towards restoration in those patients who also received Corynebacterium parvum as immunotherapy.

C. Cellular Immune Competence and Suppressor Cells

Cell-mediated proliferative responses to the mitogens Con A and PHA, and to alloantigens in MLC, were often severely depressed in lung cancer patients, and with lesser frequency in breast cancer. We worked out the methodological problems related to an accurate assessment of immunodepression with these tests, and defined a relative proliferation index, in comparison with the values for three or more normal donors tested the same day. Con A (40%), PHA (50%) and MLC (47%) responses were depressed in lung cancer patients, and these responses were also depressed in breast cancer (19%, 31%, 36%, respectively). There was some correlation of the depression with the stage of the disease, and a preliminary analysis of the data suggested its usefulness for predicting recurrence.

Studies were initiated recently to determine possible mechanisms for the observed depression. About one half of the cancer patients with immunodepression were shown to have suppressor cells active on normal third party lymphocytes. In some instances, the suppressor cells appeared to be monocytes, as was reported in studies with rodent tumors. In other instances, the suppressor cells were nonadherent, and need to be characterized further. With other depressed patients, there was no evidence for suppressor cells and this may reflect an intrinsic defect or decreased numbers of responsive lymphocytes.

D. Responses of MPBL to Autologous Tumor Cell Extracts

Lung and breast cancer patients were tested for the proliferative responses of their MPBL to autologous tumor material. This protocol suggests that reactivity would measure reactions against TAA. Sixty percent of our tests in lung carcinoma and 40% of our tests in breast carcinoma resulted in clearcut proliferation. Low concentrations of hypotonic membrane preparations worked particularly well in this system, and they have allowed serial monitoring studies. We observed that breast cancer patients would usually lose their reactivity after X-irradiation therapy. The assessments of the production of LIF in these cultures, and of the generation of cytotoxic effector cells, have been incorporated in these protocols, to allow further evaluation of the MPBL reactivity towards tumor-derived extracts. We found that cells from several breast cancer patients, when cultured with their hypotonic membrane preparation, would develop increased levels of selective cytotoxicity against the MCF-7 cell line. Further analyses of the specificity and the cells involved in these reactions are in progress.

E. Leukocyte Migration Inhibition

Leukocyte migration inhibition (LMI) appears so far to be the most potent in vitro tool in our laboratory to detect TAA on human tumors and tumor-derived cell lines. Most of our protocols included a thorough, longitudinal investigation of LMI reactivity to various tumor extracts. Results in this assay lend themselves particularly well, both for theoretical reasons, and because the same extracts could be used in both tests, to in-depth comparisons with the results of in vivo skin testing. Since Dr. J. McCoy's arrival to this laboratory, in summer 1976, he has been the person most directly involved with the developmental and logistical aspects of the LMI assays; he will deal with those in detail in his own report Z01-CBD-08522-01.

F. Clinical and Therapeutic Protocols

1. Protocols on cancer of the lung: We have been assessing the possible benefits of post-operative adjuvant immunotherapy with BCG and allogeneic tumor cells, or BCG alone in a randomized controlled trial. It is still too early to determine the effects in either immunotherapy arm of this protocol for patients who had complete surgical resection of their tumor, and more of these "Stage A" patients are being entered on this protocol.

It was quite clear, however, that "Stage B" patients with local residual tumor burden, or metastatic "Stage C" patients, accrued no benefit from the immunotherapy and this part of the study was discontinued, and data have been prepared for publication.

2. Protocol on cancer of the breast: Our study with the Medicine Branch, NCI, on adjuvant chemo-immunotherapy in cancer of the breast has been continued. Stage II patients were randomized to (1) L-PAM, (2) Cytoxan, Methothrexate, and Fluorouracil (CMF), and (3) CMF plus immunotherapy with Corynebacterium parvum. Accrual has improved and enough patients will soon be reached for data analysis.

3. Protocol on Ewing's sarcoma: Following the departure of Dr. Tom Pomeroy from the Radiation Branch, NCI, the primary clinical responsibility for this chemo-immunotherapy protocol was transferred to the Paediatric Oncology Branch, NCI, with which we have continued a good collaboration. Patients on adjuvant chemotherapy following irradiation of their primary tumor, and patients on chemotherapy for metastases to other sites were followed-up. Patients with metastases, and with "high-risk" primary lesions of the trunk and proximal segments at the limbs were randomized to additional immunotherapy with BCG and allogeneic tumor cells.

4. Protocols on melanoma patients: Our study with the Surgery Branch, NCI, on adjuvant immunotherapy in melanoma has been continued. Patients with locally recurrent lesions were randomized to (1) local injection of BCG, and (2) local injection of DNCB.

Significance for Biomedical Research and the Program of the Institute:

The capability to distinguish between NK activity and activity of immune CTL, that we developed in this laboratory, represents a very significant progress towards the identification of CTL immune to TAA in cancer patients, a step generally viewed as crucial for the better understanding of CTL-mediated reactions that may be important in vivo. In addition, based on our data in animal systems, we feel that NK cells could also play a major role in immune surveillance and rejection of tumors. Their precise characterization, and the analysis of their specificity may represent the best way to investigate this further. The findings of our laboratory that NK cells represent the same subpopulation of FcR-positive, low-affinity sE-RFC as K cells that mediate ADCC reactions, open major avenues of research aimed at characterizing potential natural lymphocyte-dependent antibodies, or at finding fundamental differences between NK and ADCC. The demonstration that high numbers of FcR-positive effector cells, active in NK and ADCC, can be generated in cultures from FcR-negative precursor cells is new altogether, and will allow better characterization of the cell surface kinetics of the FcR, and associated cytotoxic functions. This would fall under Objective V, Approach 4 of the National Cancer Plan.

The capability to generate large numbers of pure and functional T cells from any given individual opens considerable avenues for use of these cells in in vitro assays of cell-mediated immunity. Research will now have to be also started on the potential use of these cells for immunotherapy. The technique may turn out to be the cleanest approach to the purification of considerable numbers of immunoreactive T cells, and it will certainly hold a very high priority in many cancer research laboratories for the years to come. The more general ability of our laboratory to fractionate subpopulations of lymphocytes, and of the cellular components of tumors, should allow detailed direction of the role of the immune response in limiting or preventing tumor growth. These studies are also potentially useful for the immunodiagnosis of cancer and in measuring changes of immunity in response to chemotherapy or immunotherapy. This would fall under Objective V, Approach 4 and Objective VI, Approaches 1 and 4 of the National Cancer Plan.

The available techniques for the solubilization and isolation of tumor-associated cell surface antigens are important in their application to immunological monitoring, as described in this report. Such antigens may perhaps also be useful for therapy in the future. This would fall under Objective VI, Approaches 1, 2 and 4 of the National Cancer Plan.

Detection of tumor antigens and of immune reactions to human tumor-associated antigens is a major goal in tumor immunology. The development and standardization of in vitro assays for cell-mediated immunity is one of the most important aspects. Because immunogenicity cannot be studied directly in the human, the evaluation of immunologic reactivity and the antigenicity of tumor cells by those assays is of paramount importance. This would fall under Objective V, Approach 4 of the National Cancer Plan.

The question of whether immunosuppressive factors play a role in interfering with effective immunity is also very important. It appears that the stimulation of the host immune system may be useless, unless one can first rid the body of the inhibitory phenomena related to tumor cell growth. Investigation in this area would fall under Objective IV, Approach 4 and Objective VI, Approaches 1, 2, and 4 of the National Cancer Plan.

The use of various forms of immunotherapy in man has elicited considerable interest. It is imperative that the efficacy of such treatments be evaluated by clinical follow-up, and that the mode of action on the immunological system, if any, and the role of the immunological system during such therapy be clarified. This would fall under Objective V, Approach 4 and Objective VI, Approaches 1, 2, and 4 of the National Cancer Plan.

Proposed Course of Project:

This project represents our laboratory's commitment to understanding the role of the immunological response of cancer patients to the tumor and tumor progression. As has been emphasized previously in this report, the functional integrity of each cell type in the immune system, as demonstrated

by intact cell-mediated and humoral natural and immune reactions, as well as the capacity of a particular cell type to mount an effective response to the tumor, may be of paramount importance and should represent one of the ultimate goals of our research.

The priorities concerning our work with the cultured T cells will be as follows: (1) Biochemical characterization of the growth promoting factor in the conditioned media; (2) biological characterization of the effects of the conditioned media; (3) evidence for further T-cell related functions of these cells; (4) evidence for immunologic memory for antigens; (5) production of anti-T cell sera, and absorptions of anti-B cell sera; (6) development of similar cultured T cells in the mouse, in order to study their effects in vivo; (7) development of antigen reactive clones; (8) preliminary studies on the use of cultured autologous T cells for immunotherapy in patients with cancer or immunodeficiency.

The priorities concerning our analysis of the mechanism of NK and ADCC will be: (1) Demonstration of the mechanism of NK activity (development of a model for "arming"; elution and reconstitution experiments; use of an anti-Fab' reagent); (2) further analysis of the cell surface markers of NK and K cells, fresh and induced in culture (FcR; sE receptors); (3) further analysis of the specificity of NK cells; (4) generation and detection of NK and immune T cell activities in vitro; (5) physical separation of NK cells and immune T cells and their precursors; and (6) cellular lineage of NK cells.

The priorities concerning our immune monitoring of patients will be: (1) Use of separated subpopulations of cells in LMI and MLTI assays, to determine the nature of the effector cells and perhaps the need for cellular collaborations; (2) accumulation of serial immunological and clinical data, and the development of adequate statistical methods for analysis of serial data; (3) performance of simultaneous in vivo skin testing, and in vitro assays to examine the extent of correlation; and (4) preparation of larger batches of more purified soluble preparations of tumor-associated antigens for use in skin testing, and in LMI assays.

Some developments with our collaborative therapeutic protocols will include: (1) The replacement of the present lung cancer protocol, for patients with more advanced disease, by a protocol with high dose methotrexate and citrovorum factor rescue. This will be a unique opportunity to concentrate on the effect of a strong chemotherapy regimen on the various immunological tests; (2) better accrual of patients for the Stage II breast cancer protocol; (3) the intensification of chemotherapy (and use of a Laminar Flow room) for some selected patients with Ewing's sarcoma; and (4) detailed immunologic studies on patients brought to the Clinical Center as outpatients.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08522-01 LID

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Development and Use of Sensitive and Specific Assays of In Vitro Migration Inhibition for Monitoring Cellular Immunity to Tumor Antigens in Animals and Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James L. McCoy	Expert Consultant	LID NCI
OTHERS:	R.B. Herberman	Chief, LID	LID NCI
	A. Tagliabue	Visiting Fellow	LID NCI
	I. Suslov	Visiting Fellow	LID NCI
	K. Burk	Biologist	LID NCI
	J. Braatz	Senior Staff Fellow	LID NCI
	J. Weese	Guest Worker	LID NCI
	G. Bonnard	Visiting Scientist	LID NCI

COOPERATING UNITS (if any)
Litton-Bionetics, Inc., Frederick Cancer Research Center, George Washington University, National Naval Medical Center, Walter Reed Medical Center, Portsmouth Naval Hospital, University of South Carolina.

LAB/BRANCH
Laboratory of Immunodiagnosis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.50	3.50	1.00

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Development of sensitive and specific microassays of direct and indirect migration inhibition was conducted. These assays were used in guinea pig hepatocarcinoma and mouse mammary tumor virus-induced tumor models to measure reactivity against tumor-associated antigens and to monitor immunotherapy with BCG. The microassay appeared to detect a cross-reactive antigen between BCG and the guinea pig hepatocarcinoma. Microassays were used to longitudinally monitor reactivity against tumor antigens or BCG in guinea pigs bearing hepatocarcinomas and treated with BCG or mice bearing mammary tumors in attempts to see if they could be used as predicators of tumor recurrence or survival. Conventional direct capillary tube migration inhibition tests were used to measure reactivity of human breast and lung cancer, melanoma and Ewing's sarcoma patients against extracts prepared from fresh and tumor cell lines derived from these tumors. Indirect assays demonstrated interaction of patient's cells and tumor antigen cause the release of the lymphokine, leukocyte inhibitory factor. Studies were performed with indirect migration inhibition with supernatants containing leukocyte inhibitory factor.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objectives of this study are (1) to develop sensitive and specific in vitro assays of migration inhibition in detecting cell-mediated immunity reactivity to recall antigens such as purified protein derivative (PPD) and tumor-associated antigens (TAA) of normal and tumor-bearing hosts in experimental animals and man; (2) monitor antigenic activity of solubilized and partially purified TAA of animal and human tumors; (3) longitudinally monitor host reactivity against TAA, as a function of clinical course of disease in animals on BCG immunotherapy protocols and in cancer patients, to see if migration inhibition assays are predictive of disease recurrence, survival or efficacy of therapy; and (4) to measure immunologic cross-reactivity between (a) human tumors of the same or different histologic types; (b) BCG and a guinea pig hepatocarcinoma, and (c) TAA of human breast cancer and mouse mammary tumor virus and its major glycoprotein (gp52).

Methods Employed:

Antigens and lymphoid cells for migration inhibition: Crude or solubilized and partially purified antigen preparations of fresh or tissue cultured tumor and normal cells were made by a variety of procedures including sonication and nitrogen bomb disruption of cells or extraction with 3M KCl or papain. The antigen preparations were quantitated by the protein method of Lowry and aliquotted and stored at -70°C.

Peripheral blood leukocytes or mononuclear cells of humans and guinea pigs and peritoneal exudate cells of mice were used.

Migration inhibition assays: Direct capillary tube migration inhibition (LMI) assays were run with human peripheral blood leukocytes (PBL). Direct agarose microdroplet migration inhibition assays were also run with human or guinea pig PBL or with mouse peritoneal exudate cells. Finally, indirect agarose microdroplet migration inhibition tests were run with leukocyte inhibitory factor (LIF) production by human mononuclear cells tested on human polymorphonuclear cells and migration inhibitory factor (MIF) by mouse spleen cells tested on mouse peritoneal exudate cells.

Major Findings:

I. Migration Inhibition Assay Development

A. Introduction

The direct capillary tube LMI assay has been used by our laboratory for a number of years to monitor human reactivity against TAA, as will be described later. We have attempted to develop a sensitive direct micro-LMI assay that would require smaller quantities of whole blood, as the source of leukocytes, and antigens. We therefore have developed an agarose microdroplet LMI assay in the human and guinea pig which is less cumbersome than capillary tube assays to perform routinely and which requires only 2-10 ml of whole blood.

We have also been involved in developing indirect migration inhibition assays using human polymorphonuclear indicator cells with the agarose microdroplet method. Emphasis has also been placed on attempting to generate supernatants containing very high concentrations of LIF to be assayed on the polymorphonuclear cells. Finally, we were interested in determining the usefulness of the agarose microdroplet assay for monitoring the quantities and specificity of antigen preparations of animal and human tumor cells that had been solubilized and partially purified. Initial LMI tests with the assay were run with PPD as the antigen and using leukocytes from human tuberculin skin-reactive donors or from guinea pigs immunized with BCG.

B. Summaries of our progress and findings are listed below using the agarose microdroplet technique:

1. Human LMI Reactivity to PPD

Direct and indirect agarose microdroplet migration inhibition assays were performed with log dilutions (50-5 x 10^{-8} µg/ml) of soluble purified protein derivative (PPD) of tuberculin and leukocytes (4 x 10^5/droplet) from PPD skin test positive or negative individuals. Some tests were run in parallel with the capillary tube method. Inhibition of migration of leukocytes from 9/11 PPD skin test positive donors studied was observed in direct tests when employing PPD doses ranging from 1-50 µg/ml PPD. Inhibition of migration of leukocytes from some PPD skin reactive donors was obtained with as little as 5 x 10^{-3} -- 5 x 10^{-7} µg/ml (i.e., 5 nanograms to 0.5 picograms). Some inhibition of leukocyte migration was seen with skin test negative donors (presumably toxicity) with the higher doses of PPD (50 µg/ml), but generally little inhibition was observed with these donors at lower doses. Comparative experiments of the agarose micromethod and the capillary tube technique indicated that the agarose assay was more sensitive in that it could detect reactivity with 2-4 logs lower antigen concentrations. Indirect assays using supernatants of cultures of PPD triggered mononuclear cells and indicator granulocytes in agarose droplets demonstrated that a lymphokine (presumably leukocyte inhibitory factor) was being produced and suggested that cell-mediated immune reactions were operative. The results indicate the usefulness of this technique in the sensitive detection of CMI against such antigens as soluble PPD. The assay should prove useful in quantitative assessment of cell-mediated reactivity by using a wide range of antigen concentrations.

2. Guinea Pig LMI Reactivity to PPD and Crude Tumor Antigen

We were interested in seeing whether an agarose microdroplet assay of LMI could be used to measure reactivity against TAA and PPD using PBL of guinea pigs bearing syngeneic line 10 hepatocarcinomas or cured of tumors with BCG therapy. LMI assays were also performed in further investigating the possible antigen cross-reactivity between line 10 tumor and BCG. The ability to detect reactivity against PPD and line 10 tumor antigens in the guinea pig therapy model with this microassay would allow for routine longitudinal monitoring *in vitro* reactivity as it may relate to the disease status of the host.

We successfully demonstrated cell-mediated reactivity to line 10 tumor antigens or PPD with the agarose microdroplet LMI assay using peripheral leukocytes from line 10 tumor-bearing animals or animals cured of tumor with BCG. The assay was quite sensitive in that nanogram and picogram concentrations of the crude line 10 antigen and PPD were capable of eliciting strong LMI reactivity in some immune animals.

Our LMI data were also strongly suggestive of a cross-reactive antigen between line 10 tumor and BCG. Animals exposed to only line 10 reacted with PPD and others sensitized with only BCG reacted with line 10 antigen. There was a significant correlation between line 10 and PPD antigen reactivity in simultaneous LMI tests with animals sensitized with only BCG, whereas no significant correlation between PPD and syngeneic but antigenically unrelated L2C antigens was observed with these animals in simultaneous tests.

The LMI reactions appeared to be lymphokine-mediated as evidenced by indirect assays with supernatants generated by interaction of PPD with peripheral mononuclear cells from BCG immunized guinea pigs. The soluble factor in our supernatants was presumably guinea pig leukocyte inhibitory factor (LIF) since our indicator populations in the indirect assays were guinea pig polymorphonuclear cells.

The results clearly demonstrate that the agarose microdroplet LMI assay can be potentially used to monitor tumor-directed and BCG immunity in a line 10-BCG therapy model. The ability to use PBL from as little as 2-5 ml whole blood makes the assay ideal for longitudinal monitoring.

3. Monitoring Antigenic Activity of Mouse SV40 Virus-Induced Tumor Antigen Extracts

Macrophage migration inhibition assays, using the direct agarose microdroplet method, were used to monitor TAA activity of preparations of SV40-induced mKSA cells. The antigen preparations included cell-free crude membranes, papain-solubilized and NP40 detergent-solubilized membrane extracts from mKSA tumor cells. The assay was extremely sensitive and could detect migration inhibition reactivity with all three types of antigenic preparations with concentrations as low as 250 picograms protein/ml. The reactivities were quite reproducible from experiment to experiment using the same or different lots of these antigen preparations, and the reactivities were specific in that peritoneal exudate cells from BALB/c mice, immunized with antigenically unrelated but syngeneic plasmacytomas, were not inhibited by these antigens. The results demonstrated the usefulness of the assay in rapidly detecting small concentrations of partially purified TAA preparations using small numbers of immune cells.

4. Monitoring Antigenic Activity of Mouse Plasmacytoma Antigen Extracts

Cell-mediated immunity, as detected by the agarose microdroplet macrophage migration inhibition assay, was investigated using peritoneal

exudate cells (PEC) of BALB/c mice and several crude membrane (CM) and solubilized preparations of murine plasmacytomas. The assay was quite sensitive and detected inhibition of macrophage migration as low as picogram quantities of CM, NP40 detergent- and papain-solubilized preparations (CS) of ADJ-PC5 and LPC-1 plasmacytomas. The data were highly reproducible from one experiment to the next utilizing the same or different lots of the CM or solubilized extracts. Specificity studies demonstrated that ADJ-PC5 and LPC-1 plasmacytomas expressed cross-reactive tumor-associated antigens (TAA). No cross-reactivity was observed with similar extracts prepared from the unrelated syngeneic simian virus 40 (SV40)-induced sarcoma. The inhibition of macrophage migration observed was mediated by culture supernatants generated from the mixture of plasmacytoma immune spleen cells with antigens and then assayed in an indirect assay on normal PEC.

5. Monitoring Antigenic Activity of Human Breast Carcinoma and Melanoma Antigen Preparations

We are in the process of monitoring partially purified polyacrylamide gel fractions of the MCF-7 cell line derived from human breast cancer and of melanomas, prepared by Dr. Ariel Hollinshead of George Washington University. Reactivity with patients' leukocytes has been observed with these gel fractions with as little as nanogram quantities of material. Assays are in progress to determine the specificity of these reactions and to determine the fractions with the optimum reactivity, and the correlation of these results to those obtained by skin tests for delayed hypersensitivity.

6. Generation of High Concentrations of LIF for Use in Indirect Assays

In addition to developing the direct migration inhibition assay at a more micro-level, we have been interested in developing supernatants to be used in indirect migration tests that contain high levels of LIF. If supernatants containing high concentrations of LIF can be made by small quantities of mononuclear cells, this would help to miniaturize a quantitative LMI assay.

We have found that supernatants containing high titered LIF (10^{-6} - 10^{-7} dilutions still reactive) can successfully be generated with 2×10^7 mononuclear cells from PPD skin-reactive human donors in response to PPD, if the supernatants are made in 50 ml polystyrene conical tubes. Generation of supernatants with only 5×10^6 cells in the 50 ml tube or generation in smaller round bottom plastic tubes with 5×10^6 or 2×10^7 mononuclear cells yields LIF titers that dilute to only 1:2 - 1:10. The results suggest that a combination of the geometry of the tube (conical in shape) and the high cell concentration (2×10^7) is essential for the production of these high titered LIF preparations. Biochemical analysis using Sephadex G-200 columns of such supernatants indicated that the reactive molecule(s) was in the molecular weight range (69,000) of LIF as earlier reported.

II. Use of Agarose Microdroplet LMI Assays to Monitor Immunity to TAA and PPD in BCG-Tumor-Cured or Tumor-Bearing Guinea Pigs

A. Introduction

Since we were successful in demonstrating reactivity to PPD and TAA of line 10 hepatocarcinoma cells by immune guinea pigs in agarose microdroplet LMI assays, we used the assay to longitudinally monitor reactivity against the antigens in guinea pigs either cured of line 10 tumors with BCG or line 10 tumor-bearing animals. We wanted to see if the assay, used in or well-controlled BCG immunotherapy model with inbred guinea pigs, could be predictive of tumor recurrence, survival, or efficacy of therapy.

B. Experimental Design and Results

The experimental design was developed such that it was analogous to the clinical situation where patients with solid dermal tumors are treated by intratumoral BCG followed several weeks later by surgery. This design was developed in guinea pigs to achieve reactive systemic immunity for control of microscopic metastases at distant sites as well as control and elimination of regional tumor and lymph node metastases. Guinea pigs received an intradermal (id) inoculation of 10^6 (an LD-100 dose) tumor cells on day 0 followed by intravenous (iv) injection of graded concentrations of line 10 on day 6 to induce artificial metastases. These injections were such that with increasing doses per group the mortality was 30 to 100%, respectively. Local dermal tumors then were intralesionally injected with a curative dose of BCG on day 7 and these tumors along with regional lymph nodes were surgically excised on day 42. Direct agarose microdroplet LMI assays using peripheral blood leukocytes were longitudinally performed on all guinea pigs using a sonicate of line 10 (TAA) and PPD antigens on days 28 through 120 to determine if CMI reactivity was predictive of tumor recurrence or survival. LMI was predictive of disease prognosis and survival when mean values were evaluated as a function of treatment groups. Guinea pigs with large tumor burdens in groups where the mean survival was low [those animals receiving 10^6 id tumor, no BCG therapy or surgery and 10^6 iv tumor (1/43 survivors)] generally failed to become LMI-positive to either TAA or PPD. By contrast, groups having a favorable prognosis [those receiving 10^6 id tumor, curative intralesional BCG and surgery as well as low doses of artificial vascular metastases (10^4 or 10^3 cells, 20/28 survivors)] responded by LMI to TAA and/or PPD, between days 28-70, with the reactivity waning and eventually disappearing by day 96-120. These results thus suggest that LMI may be predictive of efficacy within a randomized protocol, assuming that there are adequate group sizes.

III. Use of Direct Capillary Tube LMI to Measure Cellular Immunity to Human Tumor Antigens

A. Introduction

We have previously demonstrated that the capillary tube LMI assay could measure patient reactivity against crude 3M KCl extracts of fresh breast

carcinomas and melanomas. Recent investigations were directed towards evaluating reactivity of Ewing's sarcoma and lung cancer patients against 3M KCl extracts fresh tumor tissues. We were further interested in seeing if similar extracts of tissue culture cells derived from Ewing's sarcoma, lung carcinomas, and breast pleural effusions could also serve as antigen sources in LMI assays.

B. Summaries of our findings are presented below:

1. LMI Reactivity with Ewing's Sarcoma Patients

Using crude antigen preparations obtained by 3M KCl extractions of fresh Ewing's sarcoma or of tissue culture cells derived from a pleural effusion of Ewing's sarcoma patients, assays were performed with these patients' leukocytes, patients with cancer of other sites, and normal donors. The results demonstrated approximately 60% or greater positive LMI reactivity in Ewing's sarcoma patients, as compared to less than 10% reactivity of normal donors, using extracts of either fresh or tissue cultured Ewing's sarcoma cells. A lower proportion of positive reactivity was observed in patients with breast and lung carcinoma. Further specificity tests indicated that a smaller proportion of patients with Ewing's sarcoma had LMI reactivity with KCl extracts of tissue cultured cells derived from breast cancer or fresh lung cancer cells than did patients with the homologous disease. The results indicate that many patients with Ewing's sarcoma have cellular immunity toward TAA on Ewing's sarcomas. Since all of the LMI assays were performed with allogeneic extracts, the data also suggested that different Ewing's sarcomas possess common antigens and that some breast and lung carcinomas may share some TAA with Ewing's sarcoma.

2. LMI Reactivity with Lung Cancer Patients

Direct capillary tube leukocyte migration inhibition assays were performed to determine whether lung cancer patients display cell-mediated immunity against tumor-associated antigens. The sources of antigen were 3M KCl extracts of a tissue cultured squamous cell lung tumor (CaLu-1) and a fresh pleural effusion (7661) from a patient with pulmonary adenocarcinoma. A total of 40/67 (60%) lung cancer patients reacted to 7661 as compared to 4/40 (10%) normal donors. A similar reactivity (21/38 lung cancer patients and 1/16 normal donors) was obtained with the CaLu extract. The following proportions of patients in the various histological types reacted with 7661 and CaLu, respectively: squamous, 17/28 (61%) and 7/15 (47%); adenocarcinoma 12/17 (71%) and 7/13 (54%); anaplastic, 4/8 and 3/5; and oat cell, 4/7 and 2/3. Thus, there was no histological restriction in LMI reactivity with these two extracts. Lung cancer patients with the following stages of disease reacted with the 7661 and CaLu extracts, respectively: Stages I and II, 15/20 (75%) and 9/16 (56%); Stage III, 19/36 and 9/18. Patients were also positive during relapse and within a month of death. The results suggest that lung cancer patients have CMI against common TAA on both of these extracts and these patients appear to display a similar incidence of LMI reactivity regardless of the magnitude of their tumor load.

3. LMI Reactivity of Breast Cancer Patients with MCF-7 Tissue Culture Cells Derived from Breast Carcinoma

A total of 27/32 (84.4%) breast carcinoma patients gave positive migration inhibition results on their initial tests with a 25 µg/ml protein concentration of a 3M KCl extract of MCF-7, whereas only 1/24 normal donors tested reacted with MCF-7. An intermediate incidence of reactivity (7/16) was observed with the extract when employing leukocytes of patients with melanoma, lung carcinoma and Ewing's sarcoma. In further specificity studies leukocytes of patients with breast carcinoma gave a lower incidence of LMI reactivity than did those of Ewing's sarcoma patients and lung carcinoma patients with KCl extracts of the appropriate histologic type of tumor. The results indicate that the MCF-7 cells possess a tumor-associated antigen to which many breast carcinoma patients are sensitized.

IV. Use of Direct Capillary Tube LMI and Lymphocyte Blastogenesis to Measure Cross-Reactivity Between Human Breast Cancer and Mouse Mammary Tumor Virus Antigens

Because of the potential etiologic and diagnostic implications of a potential specific immunologic relationship between human breast cancers and mouse mammary tumor virus, direct migration inhibition and lymphocyte proliferation assays were conducted with leukocytes from breast carcinoma, benign breast disease, other cancer patients, and normal donors using mouse mammary tumor virus (MMTV), its major glycoprotein (gp52), murine leukemia virus (MuLV) glycoproteins (gp69/71), a normal mouse H_2 glycoprotein and 2 human glycoproteins and fetuin (a glycoprotein of fetal bovine serum) as antigens. A total of 13/15 (83%) breast carcinoma and 3/5 benign breast disease patients reacted with MMTV, while 1/16 (6%) normal donors and 0/9 patients with other cancers reacted. Migration inhibition with gp52 was positive in 39/60 (65%) breast carcinoma, 8/15 (53%) fibrocystic and 2/11 (18%) fibroadenoma patients, 1/15 (7%) normal donors and 3/15 (29%) patients with other cancers. Specificity studies with gp69/71 preparations of Rauscher leukemia and Kirsten sarcoma virus gave approximately 40% incidence of reactivity of breast carcinoma patients, 20% with benign breast disease, 10% normal donor and 0% with patients with other cancers. No significant reactivity was observed with breast cancer patients with the mouse H_2 antigen. However, significantly more reactivity of breast cancer patients was observed with the 2 human glycoproteins and fetuin than was observed with normal donors or patients with cancer of other sites. Kinetic experiments using different harvest days and population studies with lymphocyte proliferation assays were unable to discriminate breast cancer patients from normal donor reactivity with MMTV, gp52, gp69/71 or H_2 antigens. Results suggest that many breast carcinoma and benign breast disease patients have LMI hyper-reactivity against many glycoproteins including those of MMTV and MuLV. Due to lack of restriction of LMI specificity to antigens of MMTV in both patients with malignant and benign breast disease, caution must be presently taken in interpreting the etiologic significance of these findings.

V. Indirect Agarose Microdroplet Migration Inhibition Assays Using Supernatants Generated from Mononuclear Cells of Patients with Breast and Lung Cancer

As previously described, significantly more reactivity of breast and lung cancer patients than of normal donors to the 3M KCl extracts of fresh and tissue cultured breast and lung cancer cells was observed in direct capillary tube assays. We wanted to document that the reactivity was mediated by the lymphokine, LIF. Therefore, indirect migration inhibition tests were performed with polymorphonuclear cells and supernatants generated in cultures of mononuclear cells with either MCF-7, the major glycoprotein (gp52) of mouse mammary tumor virus, or a lung cancer extract, 7661. Agarose microdroplet assays were used to test the supernatants in order to demonstrate that this assay could measure LIF activity. Greater than 50% of the breast cancer patients tested generated reactive supernatants in response to both the 3M KCl extract of MCF-7 and the gp52 antigens. A similar frequency of positive results was obtained when mononuclear cells of lung cancer patients were incubated with the 3M KCl extract of 7661. No appreciable reactivity was seen with supernatants generated by these 3 antigens when incubated with mononuclear cells of normal donors. The activity of the supernatants appeared to be due to the lymphokine, LIF, since fractionation of the supernatants on Sephadex G-200 showed the active materials to have a molecular weight in the range of LIF (69,000). Thus, the results indicate that these antigenic materials induce the specific production of the lymphokine, LIF, by cancer patients.

VI. Agarose Microdroplet Migration Inhibition with a Mouse Mammary Tumor Virus Model

We have recently initiated migration inhibition studies of cellular immunity against TAA and mouse mammary tumor virus and gp52 in a syngeneic mammary virus tumor model in C3H mice. Early reports with transplantation rejection studies suggested that C3H (C3H+) mice congentially infected with mammary tumor virus via milk transmission were immunologically tolerant to antigens of the mammary tumors, whereas C3H (C3H-) mice foster nursed and negative for the virus were immunologically reactive against such antigens.

We have been inoculating C3H+ and C3H- mice with a transplantable C3H+ mammary tumor (MT-2) which grows progressively. Spleen cells of tumor-bearing animals were harvested at various times after tumor inoculation and in an indirect assay tested for MIF production in response to a 3M KCl extract of MT-2, mouse mammary tumor virus and gp52. Both C3H+ and C3H- mice bearing the MT-2 tumor developed reactivity against MT-2 antigens and the mammary tumor virus, when the tumors were only 2-3 mm in diameter. Strong responses against the antigens were also observed in both mice substrains as the tumors reached 15-25 mm in diameter. However, as the C3H+ animals' tumors reached 35-40 mm in diameter, they lost detectable reactivity against both antigens, while C3H- animials were still reactive. Specificity of these reactivities was evidenced by the lack of reactivity of normal uninoculated animals and by the lack of reactivity of MT-2 immune mice against antigenically unrelated mouse plasmacytoma. The results demonstrate that C3H+ mice are not tolerant

to either antigens of a mammary tumor or to antigens of mouse mammary tumor virus. Further, they suggest that an eclipse of immunity as detected by the in vitro assay can occur in large tumor-bearing C3H+ mice, a phenomenon not yet seen in C3H- mice.

Significance of Research:

The ability to successfully develop direct and indirect migration inhibition microassays for measuring cellular immunity against tumor-associated antigens is of considerable interest and importance. This should permit the possible application of this assay for diagnostics of cancer or for monitoring disease progression or regression following treatment. This would fall under Objective IV, Approach 4 of the National Cancer Plan.

Similarly, it is important in longitudinal patient monitoring to have standard batches of antigen sources available for repeat testing. This has been provided by tissue culture cells derived from breast cancer (MCF-7), and lung cancer (CaLu) and Ewing's sarcoma (5838). Such cultured cells, since they do possess antigencity, are excellent candidates to be used for TAA isolation and purification. The migration inhibition assay appears to be quite useful to monitor the purification of human tumor-associated antigens from these cells. This would fall under Objective VI, Approach 1, 2 and 4 of the National Cancer Plan.

Detection of cellular immune reactivity of breast cancer patients to antigens of mouse mammary tumor virus may be valuable for the diagnosis and monitoring of breast cancer patients. Further, such cross-reactivity may ultimately shed light on the etiology of human breast cancer. This would fall under Objective V, Approach 4 of the National Cancer Plan.

Demonstration of apparent cell-mediated immune cross-reactivity between antigens of BCG and line 10 hepatocarcinoma cells may help to explain the mechanism of the immunotherapeutic activity of BCG, at least in this animal model system. Further, the longitudinal monitoring of immunity to BCG and TAA of line 10 tumors by migration inhibition during the course of a well-controlled immunotherapy trial may help provide information about the usefulness of migration inhibition assay for predicting prognosis or response to therapy.

The utilization of a murine mammary virus-induced tumor model to investigate the development of immunity against TAA and mammary tumor virus antigens as a function of progressive tumor growth may serve as a model for the monitoring of cellular immunity in breast cancer patients. This would fall under Objective V, Approach 4 of the National Cancer Plan.

Proposed Course of Project:

We plan to continue our attempts to develop microassays of migration inhibition (direct and indirect) which can be used as a practical immunodiagnostic test for possible early diagnosis of cancer as well as for serial monitoring of the immune reactivity of cancer patients. We will continue to use such assays to monitor the purification and isolation of TAAs. We will

also continue working on generating and characterizing supernatants containing high titered LIF to be used as a standard in our indirect tests involving unknown supernatants generated by patient materials. Reproducible generation of high concentrations of lymphokine may make possible the use of even small numbers (10^5 or less) of mononuclear cells in microassays. Emphasis will also be placed on biochemically characterizing the supernatants for presence of LIF.

We will continue to carefully study the specificity of reactivity of breast cancer patients against mouse mammary tumor virus antigens. It will be important to dissect apart the present reactivities now seen against MCF-7 and gp52 as well as nonvirion glycoproteins such as fetuin. It will be important to determine whether breast cancer patients have hyper-reactivity against glycoproteins in general or against a carbohydrate structure common to a variety of viral and nonviral glycoproteins. In addition, we need to determine whether these patients also have more specific reactivity against antigens on MCF-7 or gp52.

During the next year emphasis will be placed on longitudinally monitoring patients with lung and breast cancer and Ewing's sarcoma for migration inhibition reactivity against standard batches of TAA contained in extracts of homologous tissue culture cells by the direct capillary tube and possibly the agarose microdroplet techniques. The serial laboratory data will be carefully analyzed with the help of Dr. Robert Connor of the Biometry Branch of NCI, to determine correlations with clinical status and therapy. It is hoped that procedures can be developed for early detection of recurrences of disease and for indicating prognosis. In parallel, we will continue monitoring with migration inhibition in the guinea pig hepatocarcinoma and mouse mammary tumor models with various therapy protocols. Hopefully some procedures for prediction of disease regression and recurrence and survival can be obtained in these models where clinical variables can be carefully controlled.

Studies related to the specificity of the apparent cross-reactivity between BCG and line 10 hepatocarcinoma cells will be continued using microbial antigens other than BCG and other tumors such as L2C leukemia and antigenically unrelated line 1 hepatocarcinoma cells.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08517-02 LID

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cellular and Tumor Immunity in Experimental Animal Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.8	4.8	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Cellular and tumor immune responses were examined with different assays. T cell-mediated cytotoxicity against tumor-associated antigens on intact tumor cells does not require similarity at the major histocompatibility (H-2) complex (MHC). T cell-tumor cell interaction to produce migration inhibition factor (MIF) does not require H-2 compatibility. Soluble antigen can stimulate MIF production only with macrophages sharing part of the MHC with the responding lymphocytes, but when intact tumor cells are used as antigen, macrophages are not required. Different subpopulations of T lymphocytes produce MIF, depending on whether the antigen is soluble or intact. Syngeneic or allogeneic tumor cells can induce secondary cytotoxic responses against syngeneic targets. In vitro secondary cytotoxic responses require proliferation. Macrophages in tumor-bearing animals are activated to kill tumor cells. Adherent cells from peritoneal exudate, spleen and tumor can nonspecifically lyse many tumor targets. Activation of macrophages requires T cells with some inducers while in other circumstances (as in nude mice) macrophage activation can occur without T lymphocytes. Effector cells that mediate natural killer activity and those that mediate antibody-dependent cytotoxicity against tumor cells have similar characteristics and may belong to the same subpopulation of cells.

Project Description:

Objectives: The objectives of this project are: (1) to detect tumor-specific and tumor-associated antigens and to detect quantitative changes in normal antigens on malignant cells; (2) to detect cellular immune reactions to tumor antigens; (3) to develop and standardize in vitro assays to measure cell-mediated immunity to tumor antigens; (4) to correlate the findings of the in vitro assays with the growth of the tumor in the host; (5) to evaluate the diagnostic, prognostic and therapeutic significance of cellular immune responses to tumor antigens and to evaluate the effect of tumor substances (such as antigen, antibody and antigen-antibody complexes) on these cellular immune responses; (6) to measure general immunological competence of tumor-bearing animals, and to determine the relationship between this and the growth of the tumor.

Methods Employed: Host lymphoid cells are obtained from the spleen, lymph node, peritoneal cavity, peripheral blood, thymus and/or tumor of tumor-bearing, immune and control animals. A variety of normal and tumor cell lines are maintained in tissue culture. A variety of tumors induced by viral, chemical or unknown agents are maintained by transplantation in syngeneic animals. Immune and normal lymphocytes and tumor cells are stored in a viable frozen state. Inbred strains of mice are employed for several different allogeneic and tumor model systems. Immunological competence and/or cellular immune reactivity are measured in vitro by the following tests: lymphocyte stimulation by mitogens, lymphocyte-mediated cytotoxicity (^{51}Cr and $^{125}IUdR$ release), macrophage-mediated cytotoxicity, inhibition of lymphocyte-mediated cytotoxicity, production of migration inhibition factor, generation of primary and secondary cytotoxic responses, suppression of mitogen-induced proliferation, and inhibition of growth of lymphoma cells. In vivo, immunity was assessed by adoptive transfer and by challenge experiments. Animal tumor cell membrane and soluble fractions were prepared for antigenic study. Lymphoid populations were enriched or depleted of different subpopulations by iron-magnet treatment, passage over adherence columns, treatment with antisera plus complement, or adsorption on antigen or antigen-antibody monolayers.

Major Findings:

I. Cell-Mediated Immunity in the MSV Tumor System

Background

Murine sarcoma virus (MSV) when injected into normal mice produces tumors which appear 7-9 days after injection, reach peak size at 12-13 days, and regress by 21-25 days. Several different T cell-mediated immune responses can be measured in these animals. Primary cytotoxic reactivity (as detected by 4- or 18-hr ^{51}Cr release cytotoxicity assays, by 48-hr $^{125}IUdR$ cytotoxicity tests or by visual microcytotoxicity assays) peaks 14 days after injection.

In addition, production of migration inhibitory factor (MIF) and proliferation of spleen cells in response to stimulation with cell-associated tumor antigen also peaks 14 days after inoculation of virus. On the other hand, secondary cytotoxic responses can be observed in vivo after challenge and in vitro after stimulation for periods over 100 days after virus injection. Two different macrophage functions have also been reported previously in this system. Tumor-bearing mice have activated macrophages that are capable of inhibiting tumor cell proliferation (cytostasis) and of suppressing the proliferative response of normal spleen cells to PHA, Con A and LPS.

A. Influence of the Major Histocompatibility Locus on T Cell Function

Earlier studies from this laboratory have shown that cytotoxic reactivity of spleen cells from C57BL/6 mice injected with MSV 14 days earlier was mediated by T lymphocytes and was directed against endogenous type C virus antigen, designated MEV-SA-1. When cytotoxicity was tested in a 4-hr ^{51}Cr release cytotoxicity assay, there appeared to be an association between the major histocompatibility locus (MHC) and the cytotoxic reactivity, since syngeneic targets could be lysed efficiently while allogeneic targets were not. However, when the cytotoxicity assay was increased to 18 hours, reactivity was observed against targets which were dissimilar at the MHC. Cytotoxicity was not observed against syngeneic targets which did not express MEV-SA-1 nor was reactivity observed against allogeneic PHA blasts. Therefore the reactivity was specific and was not directed against alloantigens. Similar studies were performed in BALB/c mice injected with MSV. Reactivity was also detected by these effectors against syngeneic and allogeneic tumor targets carrying the appropriate antigens. However, the magnitude of the reactivity against the allogeneic target was lower than that found using spleen cells from MSV-immune C57BL/6 mice. Hence in our experimental system we have found H-2 preference rather than H-2 restriction. Other studies have provided additional information on the role of the MHC (or lack thereof) in the interaction between T lymphocytes and tumor-associated antigens. Animals that have been immunized against MSV can undergo a secondary cytotoxic response against a syngeneic target when they are challenged in vivo with either syngeneic or allogeneic tumor cells. Similarly, a secondary cytotoxic response can be elicited in vitro against a syngeneic target using as stimulators either syngeneic or allogeneic tumor cells. Monolayers of syngeneic tumor cells are capable of adsorbing out the cytotoxic reactivity against syngeneic and allogeneic tumor targets that are carrying the appropriate antigens. Finally, using an entirely different measure of T cell function, production of migration inhibition factor (MIF), we have been able to show that interaction between T cells and tumor-associated antigens can occur even when the MHC on the stimulating cell is different from that on the sensitizing cells. C57BL/6 immune T cells ($H\text{-}2^b$) can be stimulated to produce MIF by BALB/c ($H\text{-}2^d$) or A/J ($H\text{-}2^a$) tumor cells as well as by C57BL/6 tumor cells. Although the incubation time for the production of MIF is usually 48 hours, we could see very good activity even after 6 hours of incubation with either the syngeneic or allogeneic tumor cells, suggesting that this is not an in vitro sensitization to alloantigens.

In vivo protection experiments confirmed our previous in vitro data. When (C57BL/6 x BALB/c)F1 mice were immunized with the tumor line LSTRA of BALB/c origin, they resisted challenge with the same BALB/c tumor, and also with MBL-2 tumor cells of the other parental H-2 type, C57BL/6.

B. Mechanisms of Production of Migration Inhibition Factor

Spleen cells from animals immunized with MSV, obtained 9-19 days after inoculation of virus, when incubated in culture with antigen to which they had been sensitized, produce migration inhibition factor (MIF). MIF production is T cell dependent and can be stimulated by both soluble antigen and intact tumor cells. We have proceeded to study the different populations of cells which are involved in the production of MIF and to examine the manner in which they cooperate with or inhibit each other.

Since we have shown that MIF production in the MSV system is T cell dependent, it was of interest to determine which cell population produced MIF and which populations cooperate in this immune function. Using populations of immune spleen cells depleted of macrophages by different techniques, we found that such purified spleen cells were unable to produce MIF when stimulated by soluble antigen. However, the ability of these cells to produce MIF could be restored by the addition of normal, syngeneic macrophages which had been previously pulsed with the soluble antigen. Moreover, we observed that the macrophage-lymphocyte interaction was under the control of genes mapping in the MHC (H-2). Additional studies with two different strains of animals and with congenic resistant strains of the C57BL/10 mice have confirmed these findings and indicate that this cooperative function maps at either the K or I-A locus within the MHC.

By contrast, using intact tumor cells as a source of antigen instead of soluble tumor extracts, macrophages were not required in order to stimulate MIF production. Furthermore, direct lymphocyte-tumor cell interactions were not under H-2 restriction since both syngeneic and allogeneic tumor cells could be used as stimulators.

Next, we proceeded to fractionate immune spleen cell populations by 1 g velocity sedimentation in order to further analyze the mechanism by which two different types of antigens could stimulate MIF production. Using this technique we found that two different subpopulations of T lymphocytes were responsible for the production of MIF by the different antigens. One population, with a sedimentation velocity of 2.5-3.5 mm/hr, released MIF only when stimulated with soluble antigen in the presence of macrophages. The other population which had a sedimentation velocity of 4.5-5.5 mm/hr released MIF only in the presence of intact tumor cells, without a detectable requirement for macrophages. Moreover, the fraction which produced MIF in the presence of intact tumor cells was the only population which was cytotoxic in the 4-hr ^{51}Cr release cytotoxicity assay.

Finally, we have found that macrophages can interfere with the production of MIF in a number of circumstances. When immune spleen cells which

had been depleted of macrophages were mixed with increasing percentages of macrophages up to 10%, MIF production increased in response to soluble antigen. Addition of 15-20% macrophages actually decreased the MIF production. Furthermore, spleen cells from animals that had been injected with MSV 14 days earlier sometimes responded poorly to stimulation with intact tumor cells. By removing the macrophages, these spleen cells then produced high levels of MIF. When the spleen cells were stimulated with soluble antigen, removal of macrophages from the population and addition of 5-10% normal peritoneal macrophages often resulted in a better response to soluble antigen.

C. Cytotoxic Macrophages from MSV Tumor-Bearing Mice

Macrophages recovered from the spleen, peritoneal cavity and the tumor of murine sarcoma virus (MSV)-induced tumor-bearing mice strongly inhibited the proliferation of RBL-5 cells in a 48-hour growth inhibition assay (GIA). This inhibition was detected at ratios between 20:1 and 1.25:1 and was nonspecific since reactivity occurred equally well against tumor targets that do not carry antigens to which the tumor-bearing donors of the macrophages had been sensitized. On the basis of these observations we decided to investigate whether any cytolytic reactivity could be attributed to such activated macrophages, which might be complementary to the antigen-specific cytotoxicity of T lymphocytes.

To examine this possibility, we tested purified macrophages for their effects in an 18-hour macrophage-mediated ^{51}Cr release assay (MCRA). Macrophages were obtained from the spleen, the peritoneal cavity and the tumor of mice injected with MSV and from the spleen and peritoneal cavity of normal mice. Macrophages were purified by 3-hr adherence on plastic petri dishes. In some experiments macrophages were also separated from the bulk of the lymphocytes on the basis of their size using a velocity sedimentation technique. The adherent cells were characterized as macrophages by their histological appearance and by their phagocytic ability. The effector cells in the assay were not sensitive to treatment with anti-θ plus complement but were sensitive to treatment with silica and carrageenan and they could be removed by treatment with carbonyl-iron and a magnet. All these data support the contention that we are looking at macrophage-mediated cytotoxicity and that cytolytic macrophages are recoverable from MSV tumor-bearing mice. This cytolytic reactivity was detected at effector:target cell ratios which were approximately 10-fold higher than those required for the inhibition of proliferation and was nonspecific (as opposed to the T cell-mediated cytotoxicity) since it could be detected against antigenically unrelated target cells. The effector phase was T cell independent although preliminary observations using macrophages from tumor-bearing nude mice suggest a requirement for T cells in order to develop cytolytic macrophages. The highest cytolytic activity was seen with macrophages from the disaggregated tumor. In the MSV tumors, about 50-60% of the cells were macrophages while another 30-40% were T cells. These observations support the hypothesis that cytolytic macrophages may play a role in the spontaneous regression of the tumor, in addition to the important role of the immune T cells.

D. Proliferation Associated with the In Vitro Secondary Cytotoxic Response

When spleen cells from animals previously immunized with MSV that were stimulated in vitro with tumor cells carrying the appropriate antigens, they underwent a secondary response manifested by high levels of cytotoxic reactivity. In similar tests involving immunity to alloantigens or to some other tumor-associated antigens, a dramatic increase in proliferation has been associated with this cytotoxicity. However, in the MSV system it has been very difficult to detect significant amounts of proliferation after restimulation in vitro at most times after injection with the virus. In light of these facts we have examined the requirement for proliferation in the secondary cytotoxic response in vitro.

Spleen cells from mice immunized 30 days earlier with MSV were placed in culture with mitomycin C-treated RBL-5 cells and incubated for 7 days. Cytotoxicity and proliferation were assessed at daily intervals. High levels of cytotoxicity were detected at days 4, 5, and 6 while there was very little proliferation of the stimulated cells at any time interval. Suicide experiments were next performed to determine whether the secondary cytotoxic response could be eliminated by pulsing the responding cells at different time intervals with high specific activity ^{3}H-thymidine for 24-hour periods. The addition of high specific activity ^{3}H-thymidine 24-48 hours after the initiation of culture markedly reduced the secondary cytotoxic response. If cold thymidine was added at the same time as the isotope, there was no inhibition. Addition of the labeled thymidine during the final 72 hours of culture had little or no inhibitory effect on the development of cytotoxicity. Therefore, using this technique we have shown that cell proliferation is required for the generation of secondary cytotoxicity from the spleens of mice infected with MSV.

E. Kinetics of the Primary Cytotoxic Response to MSV

Previously we have reported that the cytotoxic reactivity of spleen and lymph node cells from C57BL/6 mice injected with MSV peaked at 14 days in the 4-hr ^{51}Cr release, the 48-hr ^{125}IUdR release, and the 48-hr microcytotoxicity assays. Two other laboratories using the microcytotoxicity assay have reported that there is a peak in reactivity 7-9 days after injection, a decrease to very low levels at 14 days, followed by a second increase in reactivity which persisted at least until 30 days. The only apparent differences between our work and that from the other laboratories was a) different targets were employed in the assays, and b) one lab used a different strain of animals. We have reexamined this problem using the ^{125}IUdR release cytotoxicity assay, but employing BALB/c mice immunized with MSV as a source of effector cells and Ha-2 cells as the target, thus more closely approximating the work of one other laboratory. Our results agree with our previously published data showing a peak reactivity 14 days after inoculation. In addition, we have reconfirmed and extended our observations concerning the lack of H-2 restriction in the MSV system by demonstrating reactivity against tumor targets that do not share the same MHC as the sensitizing cells. The reasons for the disparate results regarding the kinetics of the reactivity are still not known.

II. Activation of Macrophages in Other Systems

A. Macrophage Activation in Nude Mice -- Suppressor and Growth Inhibitory Activity

Experiments were performed to determine whether T cells are required for the activation of the macrophages which inhibit the proliferation of tumor cells and which can suppress the mitogen response of normal spleen cells. Spleen cells from nude mice injected with Corynebacterium parvum (C. parvum) were able to inhibit the growth of RBL-5 tumor cells. However, this inhibition was only about 50% of that seen with spleen cells from normal mice injected with C. parvum. Studies using various doses of C. parvum have shown that the response of normal mice was dose dependent and that doses under 1.4 mg/mouse given intraperitoneally did not induce growth inhibitory macrophages in the spleen. In contrast, preliminary studies indicate that the response of nude spleen cells to C. parvum was not dose dependent and that 0.14 mg/mouse IP was as efficient as 2.1 mg/mouse IP in generating cells which were growth inhibitory. Initial studies using the suppression of mitogenic stimulation assay show that nude spleen cells from C. parvum injected mice are not as efficient as normal spleen cells from C. parvum injected mice in inhibiting the PHA stimulation of normal spleen cells. In contrast to the reduced activity in growth inhibition and in suppression of PHA responses, nude spleen cells from C. parvum injected mice (1.2 mg/mouse IP) could suppress the proliferative response of nude spleen cells to the B cell mitogen LPS as efficiently as spleen cells from normal mice injected with the same dose of C. parvum. As was found in the growth inhibition assay, the nude suppressor activity generated by C. parvum was not dose dependent whereas that of normal mice was.

Studies are now in progress to compare these macrophage activities in spleen cells of normal and nude mice injected with MSV.

B. Macrophage Suppression in the Mammary Tumor Virus System (MTV)

Studies have been undertaken in a mammary tumor virus system to investigate whether a correlation exists between the spontaneous appearance of mammary tumors in vivo and the appearance of growth inhibitory macrophages as measured by the in vitro growth inhibitory assay (GIA). Utilizing 5 different tissue culture lines as targets (RBL-5, RAJI, and 3 different MTV cell lines - L8a, CRMT, $34I_{c1}$ 101_{c1} E_{c1} 12), no difference was observed between the ability of spleen cells from MTV- mice and spleen cells from MTV+ mice, either with or without tumors, to inhibit growth of the target cells. The inability to see a significant difference may be due to the high levels of nonspecific activation found in control mice which must be 4-5 months of age in order to be age matched with the tumor-bearing mice. We are now setting up experiments with transplanted mammary tumors in C3H mice. Hopefully these mice will be more practical for studies of macrophage suppression in the MTV system.

III. Correlation of Natural Cytotoxicity with Antibody-Dependent Cell-Mediated Cytotoxicity Against Mouse Tumor Cells

Natural killer (NK) cells that are capable of killing a wide range of tumor targets and cells capable of killing antibody-coated tumor targets (K cells) have been found in many strains of unimmunized mice. Using a long-term (18-hr) ^{51}Cr release cytotoxicity assay, we have performed parallel studies on the distribution and nature of the effector cells mediating NK and ADCC activity.

Both activities were found to have the same age distribution. In newborn and weanling animals the reactivity was usually low. NK and ADCC cytotoxicity showed peak reactivity at about 5-8 weeks of age and then there was a decrease after 10 weeks of age. Among different strains of conventional, thymus-bearing mice a good correlation was seen in tests for NK and ADCC activities against the same target cell (RBL-5 tissue culture). CBA and C3H/f mice had high levels of reactivity, C57BL/6 and BALB/c had intermediate levels and SJL had low levels. As with NK reactivity, young nude mice with random bred, BALB/c or C57BL/6 genetic backgrounds had high levels of ADCC activity. In addition to the overall correlation among groups of mice of different ages or strains, there was a fairly good correlation in levels of NK activity and ADCC among individual mice. Those animals with high cytotoxic reactivity in ADCC also had high NK activity; the same was true for those animals that had low activity.

The NK and K cell reactivities were not restricted to the spleen but were present in other lymphoid organs as well. Effector cells from the spleen and peritoneal cavity were shown to be very reactive; cells pooled from axillary, inguinal and mesenteric lymph nodes or cells from bone marrow had lower, but significantly positive reactivity. Thymocytes were found invariably to be nonreactive in both tests.

Several different in vitro and in vivo manipulations have been found to influence the level of NK and ADCC activity in a similar fashion. Inoculation of older mice (that have very low levels of both activities) with lymphocytic choriomeningitis virus or Corynebacterium parvum resulted in an increase in NK activity as well as ADCC activity. On the other hand, both reactivities were found to be depressed in MSV tumor-bearing nude mice. The sensitivity of NK and K cells to irradiation in vitro was similar. Doses of 500 R or less had little effect; however, with increasing doses of irradiation there was a corresponding decrease in both NK and ADCC activity.

The effector cells in both tests seemed to have similar surface markers and other characteristics. The NK and K cells were nonphagocytic and nonadherent. Adsorption of spleen cells on EA monolayers to remove Fc receptor-bearing cells resulted in a moderate decrease in both K and NK cell reactivities. It should be pointed out that depletion was only partial since it is difficult to remove mouse Fc receptor-bearing cells by the available procedures.

There have been some consistent differences between the characteristics of the NK and K cells. NK activity in a short-term assay was trypsin sensitive. However, NK activity in the long-term assay and ADCC were not affected by trypsin when effector cells from CBA mice were employed, but when effector cells from nude mice were used, NK activity decreased while ADCC did not. In addition, NK activity was found to be relatively more labile upon short-term incubation at 37°C than was ADCC.

Significance to Biomedical Research and the Program of the Institute:

The immune response to neoplasia is very complex and involves not only the interaction between host effector cells and tumor cells, but also the interaction between and among the various subpopulations of leukocytes. Some cells may serve to amplify or suppress the immune response to tumor growth. We have been studying these interactions on several levels, both *in vitro* and *in vivo*, in order to try to determine the role of the immune response in limiting or preventing tumor growth.

Recently there have been reports from several laboratories that the interaction between sensitized T lymphocytes and target requires similarity at the major histocompatibility locus (MHC). Our work in the MSV system using two different assays of T cell-mediated function demonstrates that similarity at the MHC is not essential for interaction between tumor cells and T cells. For several reasons this type of information is very important. First of all, when constructing models for the T cell receptor, interactions in which the MHC does not play a role must also be taken into account. Second, these results would also suggest that in the *in vitro* testing which is done to investigate T cell-mediated anti-tumor reactivity in man, the source or antigen, either as stimulator or as target, does not have to share the MHC with the effector cell. Finally, it appears (based on these results) that there are tumor-associated antigens which are shared between different strains of mice and even between different species, and which can be responded to by T cells. This would suggest that these tumors could be used as a source of antigen for immunization to protect against tumor challenge or for immunization as part of an immunotherapy regimen.

Macrophages and T lymphocytes interact with each other in both directions: (a) macrophages stimulating T cell functions, and (b) T cells assisting in the activation of macrophages. We have been investigating immune responses associated with both types of interactions. In addition, macrophages are capable of suppressing the immune response through their interactions with T cells. Hence there is a delicate balance among the helper functions of the macrophages, the macrophage suppressor activity, and the activation of macrophages by the T lymphocytes. Understanding all these interactions, what influences them and how they can be modulated to the benefit of the host will give us information that we can use to influence the growth of a neoplasm.

In order to obtain a clearer understanding of the events which influence the growth of a tumor, we have sought to determine the nature of the immunological events taking place within the tumor. Two different effector

mechanisms aimed at the destruction of the tumor have been identified: T cell-mediated cytotoxicity and macrophage-mediated cytotoxicity. The latter can manifest itself either as a lytic event or as cytostasis of tumor proliferation. It would appear that these two mechanisms for dealing with the tumor should be maximized within the host. Therefore, by developing in vitro assays for these different functions, we are able to assess their relative magnitude and, therefore, when the host is treated in an attempt to magnify these functions, these tests can assess the efficacy of treatment.

Immune T lymphocytes, when stimulated with the sensitizing antigen, respond with the production of migration inhibition factor (MIF). We have shown that there are two subpopulations of T lymphocytes which are capable of producing MIF, one in response to soluble antigen and the other in response to intact antigen. The T lymphocytes which respond to intact antigen appear to be very similar to the cells which are cytotoxic. At the moment it is not clear if there are any other differences in these two subpopulations. Many different subpopulations of T cells have been described. We have now been able to subdivide these cells based on the form of the antigen to which they respond. This could be very important with respect to their role in vivo.

We have described the presence of suppressor and growth inhibitory macrophages in the spleen and tumors of MSV injected mice. These cells could be very influential on the outcome of tumor growth. Because of this potential we have begun studies to determine the role that these cells might play in other tumor systems.

T cell-mediated cytotoxicity is a powerful arm of the immune system which appears to cause regression of tumors and influence the course of tumor growth. For this reason, we have continued studies on the various techniques for measuring primary cytotoxicity and on the events associated with the secondary cytotoxic response. The finding that only a very low level of proliferation can be detected from cells that are undergoing a secondary response in vitro, even though that proliferation is essential, raises important questions with regard to the importance of the high levels of proliferation observed in most systems.

Finally, two other types of cell-mediated cytotoxicity, natural killer (NK) activity and antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells, may also have very important roles in some tumor immune response. The finding that the cells which mediate these two functions may be identical, or perhaps come from the same subpopulation of lymphocytes, suggests that the same mechanism of target-cell recognition in ADCC (antibody, through attachment to the Fc receptor) may also play a role in target cell recognition in NK activity (perhaps through arming). Furthermore, it consolidates some of the effector cell functions which had previously been attributed to different cells. It also gives a functional role to some of the cells which had previously been described as null cells. Several other effector functions which had been attributed to the same class of cells should likewise be examined for their relationship to this subpopulation of cells.

Proposed Course of the Project:

During the next year many of the above mentioned projects will be continued and, in addition, some new projects will be initiated.

Investigations into the role of the MHC in the interaction between T lymphocytes and tumor cells will continue. Initially the observations concerning the cross reactivity have been made using C57BL/6 mice. We will attempt to look at several other strains of mice to determine the frequency of these cross reactions. In addition, we plan to examine the possibility that there are different subpopulations of T cells which are mediating the reactivity against different targets. Finally, several types of in vivo experiments will be performed (such as adoptive transfer, immunization) to determine what role the MHC has in protection in vivo against tumor challenge.

The interactions between different subpopulations of leukocytes continue to be of interest. We will expand our studies on the role of the macrophages in the augmentation or suppression of the immune response. These areas of study will be: role of the macrophage in the production and suppression of production of MIF and the role of the macrophage in the suppression of the immune response (especially in vivo). In addition, we will examine the role of macrophage subpopulations in the various aspects of immune reactivity. will build on the findings that we have made previously concerning the two subpopulations of macrophages found in the tumor and, in addition, look at the effect of macrophages from other sites within the animal on different immune functions. We will continue to use fractionation techniques (such as 1 g velocity sedimentation) to enrich for subpopulations of cells. We also hope to look at some of the mechanisms of action involved in macrophage activity.

The host response to tumor cell growth involves several different effector populations. Among these are T cell-mediated and macrophage-mediated cytotoxicity as well as natural killer activity and antibody-dependent cytotoxicity. We will continue to examine the role of these immune mechanisms, as well as the role of several of the immune mechanisms discussed above, in the tumor immune response. This will be performed on several fronts: (a) by the examination of the effector cells present in the tumor, (b) by determining the mechanism by which the cells enter the tumor and how they become sensitized or activated, and (c) by comparing the immune mechanisms which are operative in regressor versus progressor animals so as to determine the variables which make the difference.

Studies on the secondary cytotoxic response will be continued. Emphasis will be placed on correlating the secondary response observed in vivo or in vitro with the ability of the animal to resist challenge at different times after immunization. Since the protection wanes, it will be a good opportunity to see what is the correlation between these events.

Recently, it has been possible to place normal T cells into continuous culture for extended periods of time. We plan to initiate cultures of normal T lymphocytes from animals that have been immunized with MSV. If we are

successful, we will then evaluate the immunological function of these cells, in vitro and in vivo. This could provide a very powerful tool for the examination of T cell receptors and for studying the possibility of utilization of these types of cells for immunotherapy and other types of immunological transfer.

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LABORATORY OF IMMUNOBIOLOGY

Introduction

I. Cell Mediated Killing of Tumors

- A. Metastases
- B. Chemotherapy, Surgery and Immunity
- C. Glucan and Trehalose Dimycolate (Cord Factor)
- D. "Educated" Lymphocytes
- E. Immunosuppression and Tumor Resistance

II. Complement Mediated Killing of Tumors

- A. Modulation of Cell Susceptibility to Killing
- B. Interaction of Cells and Complement
- C. Chemotherapy

III. Immunologic Interactions of Host and Tumor

- A. Macrophages
- B. Lymphotoxin
- C. Basophils in Inflammation

SUMMARY

Annual Report of the Laboratory of Immunobiology, National Cancer Institute
July 1, 1976 to September 30, 1977

INTRODUCTION

The Laboratory of Immunobiology, organized on July 4, 1976, consists of three of the four sections that formerly comprised the Biology Branch in the Division of Cancer Cause and Prevention (DCCP). The fourth section, Cytogenetics and Cytology, has remained in the DCCP and is now the Biology Branch under Dr. DiPaolo's leadership. While, the laboratories of the Biology Branch and of the Laboratory of Immunobiology are still in proximity and interactions between the two groups still occur, unavoidably, the extent of this collaboration, which contributed greatly to progress in the Cytogenetics and Cytology Section, has decreased significantly. The three sections comprising the Laboratory of Immunobiology are: Cellular Immunity, Humoral Immunity (formerly Immunochemistry) and Immunopathology (formerly Tumor Antigen). The members of these sections, through their studies of tumor immunology, continue to make significant contributions to the goals of the National Cancer Program. Methods are sought to kill tumor cells in vivo and in vitro and to overcome the possible suppression of host immunity by tumors. In the Cellular Immunity Section attempts are being made to delineate the limits of immunotherapy in the treatment of experimental and naturally occurring metastatic cancers. It had been established in previous studies that a transplanted hepatoma growing in the skin of syngeneic guinea

pigs (host and tumor are genetically compatible) as well as microscopic regional lymph node metastases could be eradicated by a single intralesional injection of living (or a specially prepared fraction of) BCG. Attempts are now being made to apply this treatment alone and in combination with other forms of treatment to more advanced stages of disease. Purified fractions of BCG as well as defined agents unrelated to BCG are being tested for anti-tumor activity. Lymphocytes are being "educated" in vitro to become killers of tumor cells. Suppressor cells are being studied as possible modulators of cancer immunotherapy. Naturally occurring cancers in large outbred species are being sought and studied as guides that may yield information more applicable to cancer than that provided from studies with small, inbred rodents with artificially induced or transplanted often non-metastasizing tumors. In the Humoral Immunity Section it was found that line 10 cells, ordinarily resistant to complement mediated killing, were rendered susceptible to immune cytotoxicity by pretreating them with certain metabolic inhibitors, chemotherapeutic agents and enzymes. Lipid synthesis and not the synthesis of other compounds correlated with resistance. Inhibition of lipid synthesis correlated with susceptibility. Studies of the fundamental mechanisms of complement mediated cell killing have uncovered a clue that may help understand why line 10 guinea pig hepatoma cells were more susceptible to the toxic action of human complement than to that of guinea pig complement. Certain chemotherapeutic drugs that rendered line 10 cells susceptible to complement mediated toxicity were effective in vivo against the transplant and microscopic metastases if administered intralesionally. Therapeutic potency of these drugs did not necessarily correlate with their ability to destroy normal tissue. Under certain conditions animals cured by intralesional chemotherapy developed resistance against challenge with line 10 tumor. In the Immunopathology Section studies are continuing on the interaction of tumor cells and host's immune system. Studies that may elucidate tumor and host interactions include macrophage mediated tumor cell killing, chemotaxis, lymphotoxin mediated cell killing and the accumulation of basophils at sites of inflammation.

I. CELL MEDIATED KILLING OF TUMORS

A. Metastases. Patients are sometimes encountered with early metastases to two lymph nodes. This situation was mimicked in guinea pigs by injecting tumor cells into the skin above the thoracic spine. In this way metastases were produced in the superficial axillary lymph nodes on both sides of the animals. Intralesional BCG eliminated the primary tumor and the tumor cells in both lymph nodes. Animals with a primary tumor and microscopic metastases in only one lymph node were cured by BCG into the skin adjacent to the dermal tumor between it and the lymph node provided the dermal tumor was subsequently excised. Animals with bilateral metastases were cured by a similar procedure if BCG was applied adjacent to the tumor bilaterally but were not cured if BCG was injected only on one side. Various sites of lymph node metastases were established by varying the intradermal site of the challenge inoculum. Intradermal BCG cured animals with metastases at different sites only when the adjuvant was injected within the area of lymphatic drainage of the primary tumor.

Previous studies indicated that excision of the dermal tumor, palpable draining lymph nodes and the next non-palpable lymph nodes in the chain cured a significant proportion of animals. This observation was the basis of a model study of minimal residual disease. Animals with growing dermal tumor transplants and palpable regional lymph node metastases each received an injection of BCG into the skin tumor. One week after BCG administration the skin transplant and the regional lymph nodes were removed leaving minimal residual disease in the next lymph nodes. All animals treated in this way died with widespread lymph node metastases. BCG followed by excision of the palpable nodes as well as the next nodes in the chain cured a significant number of animals but no more than the number cured by radical node dissection alone. Passive transfer (adoptive immunization) of leukocytes from immunized tumors also failed to influence minimal residual disease. A variety of attempts to treat "advanced" disease have failed. Advanced disease in guinea pigs challenged in the flank consists of the dermal transplant, palpable metastases in the superficial distal axillary lymph nodes and microscopic metastases in the proximal axillary lymph nodes.

B. Chemotherapy, Surgery and Immunity. Chemotherapeutic agents that are effective when given intralesionally at a time when only microscopic metastases are present, fail to cure if treatment is given when there are palpable metastases.

Strain 2 guinea pig fibrosarcomas derived from embryo cells transformed in vitro were not susceptible to BCG immunotherapy. Animals with one of these transplanted fibrosarcomas could not be cured by the intralesional injection of adriamycin.

Removal of line 10 tumor by radical surgery 8 days after intradermal transplantation of 10^6 tumor cells did not lead to development of resistance to contralateral tumor challenge, whereas radical surgery 16 days after tumor implantation leads to resistance to contralateral challenge. Resistance to tumor challenge was not found in animals that did not undergo surgery and was weak in animals with small tumor recurrences. Resistance to contralateral challenge was detectable within 7 days after radical surgery and persisted for at least 120 days. Studies are underway in an effort to determine if concomitant immunity to intradermal challenge is detectable during early stages of tumor growth or when a lower initial tumor inoculum is used. The interaction of host "immunity", as manifested by resistance to tumor challenge following radical surgery, with immunoadjuvant administration during advanced stages of malignant disease is being investigated.

C. Glucan and Trehalose Dimycolate (Cord Factor). The therapeutic potential of glucan was studied using several well defined syngeneic animal tumor models. One guinea pig tumor and four mouse tumors were studied. Injection of glucan directly into guinea pig tumors had no significant therapeutic effect; whereas injection of living BCG under the same conditions was effective. Neither BCG nor glucan were active against the guinea pig hepatoma when administered intravenously. A combination of endotoxin with

a fraction of mycobacteria related to cord factor in mineral oil emulsion was effective in treatment of the line 10 cavine tumor but a similar emulsion in which glucan was substituted for endotoxin was ineffective. Administration of glucan intralesionally or intravenously was ineffective against the murine tumors. Previous claims of glucan induced anti-tumor activity against the B-16 murine melanoma were not confirmed. Administration of BCG intralesionally or intravenously was tested against the two murine fibrosarcomas and was found to be effective against one of them.

Living BCG, killed M. tuberculosis cells or BCG cell walls augmented the immunogenicity of lyophilized tumor cells (line 10). Effective vaccines contained living BCG and lyophilized line 10 cells, or mycobacterial cells or cell walls attached to oil droplets and lyophilized line 10 cells. Protection against the challenge tumor was evident 14 or 21 days after one administration of either vaccine.

BCG cell walls were compared to trehalose-6,6'-dimycolate (TDM) in admixture with endotoxin (ET) for ability to cause regression of a transplanted guinea pig hepatoma. On a weight basis cell walls were at least as active as ET-TDM. Toxicity of ET and ET-TDM were greater than that of cell walls as judged by weight loss after intravenous administration to normal guinea pigs.

Injection of emulsified TDM into transplants of an established, syngeneic murine fibrosarcoma induced complete regression of tumors. The proportion of animals showing complete tumor regression depended on the amount of oil in the emulsion. The anti-tumor activity of the TDM emulsion containing the highest concentration of oil was equivalent to that of living BCG.

Studies in mice and guinea pigs suggested a connection between the granulomatous response and the anti-tumor activity elicited by living BCG, by BCG cell wall vaccine and by emulsified TDM. The following studies were undertaken to facilitate future investigations on the possibility that the anti-tumor activity of mycobacterial vaccines required granuloma formation.

Emulsions of TDM made with various concentrations of oil and Tween were tested in mice for granulomogenic activity. The intensity of the granulomatous reaction evoked in the lungs by TDM administered intravenously in mineral oil/water emulsion depended on the number of large sized (>8μ) oil droplets in the emulsion. Among the factors that determined droplet size and number were amount of oil, concentration of Tween and the method of emulsification. For a given concentration of Tween, granulomatous activity of TDM emulsions increased with increasing oil content. Conversely, emulsions with the highest activity were obtained with low concentrations of Tween (<0.04%). Emulsions made by grinding were more active than those made by ultrasonication. The cellular reaction almost completely subsided 21 days after TDM administration. Emulsions prepared by ultrasonication may be stored for at least 7 days at -20°C or heated at 70°C for 20 minutes without obvious changes in appearance or activity.

Under the above conditions emulsions prepared by grinding were partially separated.

Emulsions of TDM made with various amounts and types of oil were tested for toxicity in mice. The toxicity of TDM administered intravenously in mineral oil/water emulsion depended on the size of the oil droplets. A highly toxic emulsion (9% oil) contained many (~20%) large oil droplets (>8μ). Emulsions (1-3% oil) with little or no toxicity contained very small oil droplets (less than 1μ) and very few, if any, larger oil droplets. Emulsions of TDM made with peanut oil or olive oil were much less toxic than those made with mineral oil. Results of these studies do not lend support to the hypothesis that granulomagenic activity or toxicity are required for the anti-tumor action of mycobacterial vaccines.

D. "Educated" Lymphocytes. Attempts to sensitize normal strain 13 guinea pig lymphocytes against syngeneic tumor cells in cell culture have continued. Functional sensitization in vitro was detected by the ability of the cultured lymphocytes to damage target cells. The tumors used were strain 13 guinea pig hepatomas and one fibrosarcoma. A reliable assay for the determination of target cell damage by in vitro sensitized lymphocytes has been established. This assay is based on radioactive labelling (with ^{51}Cr) of the surviving target cells following their coculture with effector lymphocyte populations.

It was found that strain 13 lymphocytes cultured in medium containing heterologous fetal calf serum even in the absence of stimulator tumor cells, developed the capacity to damage indiscriminately various tumor target cells as well as normal fibroblasts. These findings emphasize the problems involved in the use of heterologous serum (usually fetal calf) in in vitro model systems aimed at the induction of specific anti-tumor cell mediated immunity. They demonstrate the need to find ways for distinguishing specific anti-tumor directed cytotoxicity from "natural killer" activity.

A major step towards such discrimination was taken by demonstrating that effector cells could be generated efficiently in vitro by culturing lymphocytes with syngeneic tumor cells in the presence of fresh autologous serum. Under those conditions the "natural killer" activity could be eliminated as evidenced by the inability of tissue culture medium alone or normal fibroblasts to induce the development of effector cells. Effector cells induced by syngeneic hepatoma cells damaged hepatoma target cells but not normal fibroblasts.

E. Immunosuppression and Tumor Resistance. Studies were undertaken to determine whether a relationship existed between the immunogenicity of tumors and their ability to induce host immunosuppression. For example, one can imagine a situation where induction of immunosuppression soon after tumor inoculation would result in failure of the host to develop tumor immunity. Immunosuppression was detected by the ability of spleen cells from animals with tumors to suppress the proliferative response of normal spleen cells to the mitogen concanavalin A in vitro. Two chemically-induced mouse fibrosarcomas were studied, one of which (1023) was strongly immunogenic while the other (1063) was weakly immunogenic in vivo. The

weakly immunogenic (1063) tumor induced the appearance of splenic suppressor cells that could be detected as early as three days after tumor inoculation, while with the strongly immunogenic (1023) tumor, suppressor cells were first detectable only twelve days after tumor inoculation. The suppressor cells in this system were found to be non-T, plastic-adherent and phagocytic cells, (probably macrophages). Studies are now in progress to determine whether the early appearance of suppressor cells induced by the 1063 tumor has any biological role in preventing development of immunity to this tumor in vivo.

II. COMPLEMENT MEDIATED KILLING OF TUMORS

A. Modulation of Cell Susceptibility to Killing. The primary purpose of these experiments was the elucidation of cellular events that make a tumor cell resistant or susceptible to the cytotoxic action of complement (C). Past experiments have shown that susceptibility of the cells to C killing can be increased by treatment of cells with certain drugs, metabolic inhibitors and chemotherapeutic agents and with proteolytic enzymes. Treatment of cells with hormones led to an increased resistance to killing by C. During the last year we have greatly expanded our understanding of some of the mechanisms whereby these changes are induced in the cells.

Increase in the susceptibility of drug-treated cells to humoral immune attack appeared to be correlated with complex lipid and not with DNA, RNA and protein synthesis. Treatment of cells with heat (42 to 43°C), low doses of x-ray or brief exposure to metabolic inhibitors did not increase their susceptibility, did not affect lipid synthesis, but did inhibit DNA, RNA and protein synthesis. Inhibition of synthesis of macromolecules such as mucopolysaccharides, polysaccharides, or lipids de novo did not correlate with increased susceptibility. Furthermore, preliminary studies of antibody-C mediated attack on tumor cells showed increased synthesis of complex lipids following addition of GPC (which did not kill the cells) but not HuC (which killed the cells). Identification of lipids synthesized suggested the involvement of phospholipids.

The metabolic requirements for hormones to be effective in rendering tumor cells resistant to antibody-C mediated killing were that the cells have at least RNA and protein synthesis intact. No absolute requirement for intact DNA synthesis was detected. The resistance was not due to the physical binding per se of hormones to the cell. Hormone treated cells allowed to revert to a susceptible state were not rendered resistant when treated again with the same hormone but were rendered resistant when treated with a different hormone. This refractoriness to re-exposure was lost upon prolonged culture in medium free of hormone. Preliminary results indicated hormone treatment may increase complex lipid synthesis. Polypeptide hormones appeared to increase DNA, RNA and protein synthesis while glucocorticoid hormones depressed synthesis of these macromolecules.

Treatment of line 10 cells with proteases increased their sensitivity to killing by antibody and GPC. Following enzyme treatment the line 10 cells bound less complement fixing antibody than untreated line 10 cells despite being sensitive to killing. There was no significant difference in the ability of antibody-sensitized, enzyme pretreated, line 10 cells and their untreated counterparts to remove C1, C4 or C2 from fluid phase GPC. However, the supernatants from line 10 cells treated with proteolytic enzymes were found to contain material that inhibited the action of GPC. This anticomplementary action was not found in supernatants containing enzyme alone or supernatants from line 10 cells treated with a variety of enzymes ineffective in rendering the cells sensitive to antibody-GPC attack (e.g. DNase, neuraminidase, RNase). The intact line 10 cells were not found to be anticomplementary. The development of a cytotoxic assay based on the release of ^{125}I from $^{125}IUdR$ prelabelled cells allowed experiments in which enzyme pretreated and untreated line 10 cells could be mixed. These experiments demonstrated that killing occurred only when proteolytic enzyme treated cells were mixed with antibody and complement. No evidence for innocent bystander killing was found.

B. Interaction of Cells and Complement. The purpose of these experiments was to elucidate the nature of the cytotoxic action of complement. Line 1 cells coated with anti-Forssman antibody were killed only on addition of the entire sequence of complement components (1-9). Addition of the complement components 1 through 8 to line 1 cells resulted in a nonlethal, reversible damage to the cells as measured by the uptake and release of ^{86}Rb. Studies of ^{86}Rb release from line 10 cells on addition of antibody and GPC demonstrated significant levels of ^{86}Rb release without trypan blue uptake. Similar levels of ^{86}Rb were released from line 10 cells by antibody and HuC but in this case approximately 30% of the cells were stained with trypan blue. To further analyze the difference in efficiency between HuC and GPC the effect of adding HuC components to GPC was tested. Combinations of GPC with human sera deficient in C2, C5, C6, C7 or C8 suggested that GPC could provide the initial complement components for killing of antibody sensitized line 10 cells but that the later acting complement components in GPC were not as effective as those in HuC. Methods are being devised to measure the quantity of the later acting components of GPC or HuC bound to tumor cells. In order to develop such assays, rabbit antibody to GP or Hu 5, 8 and 9 have been produced; these reagents would permit the analysis of the terminal stages of immune hemolysis for understanding their mechanism of action.

C. Chemotherapy. The purpose of these experiments was to analyze the mechanisms whereby intralesional treatment of tumors with drugs cures animals. Treatment of 7 day line 10 tumors in vivo with several chemotherapeutic drugs was effective in causing tumor regression, eradication of metastatic tumor and development of tumor specific immunity. These effects were dependent upon dose and time of administration of the drugs. Treatment was less or not effective if started at day 14 or when draining lymph nodes were 6-7 mm in diameter (approximately 21-35 days after tumor inoculation).

Combination of local surgery with non-intralesional chemotherapy on 7 day tumors was not effective in curing animals. Injections of drugs were given between the draining node and surgery site or on the opposite flank following excision of the growing dermal transplant.

Histological examination of tumor sites and draining nodes following intralesional chemotherapy with adriamycin or DTIC were performed. Adriamycin but not DTIC was effective in causing tumor regression and eradication of tumor cells in draining nodes. Both drugs caused massive damage to the connective tissue and tumor cells. Tissue histiocyte infiltration with adriamycin treated tumors appeared to be less than that observed in the DTIC treated tumor. While it is clear that chemotherapeutic drugs promote complement mediated killing of tumor cells in vitro, there is no evidence that the action of these agents against tumors in vivo requires the participation of complement.

III. IMMUNOLOGIC INTERACTIONS OF HOST AND TUMOR

A. Macrophages. Macrophages have been reported to be important in host defense against neoplastic disease. These cells, or a functionally activated subpopulation, have antitumor activity in vitro. Mice that received intraperitoneal injections of BCG developed a chronic infection and tumoricidal macrophages could be recovered by 1 week and persisted for more than 40 weeks. The tumoricidal cells were localized at the site of BCG challenge (peritoneal cavity). Mice infected intradermally did not develop cytotoxic peritoneal cells, although the mice became immune to BCG antigens. Intraperitoneal injection of soluble BCG antigens (PPD) into these mice induced tumoricidal macrophages. Cytotoxic cells were found by 9 hours after antigen injection and persisted 2 days. Studies with a cytochemical marker for peroxidase showed an increase from a very low to a very high percentage of peroxidase-positive macrophages after PPD challenge. The peroxidase-positive cells are those that have recently migrated into the peritoneal cavity. These newly immigrated cells were not sufficient for tumor cytotoxicity, since peroxidase-positive cells induced by irritants were not cytotoxic. Induction of cytotoxic macrophages by antigen required both a macrophage precursor cell and a specific immune response.

Peritoneal macrophages from normal mice were activated to become cytotoxic after adoptive transfer in vivo of immune spleen cells and specific antigen. Similarly, incubation in vitro of peritoneal macrophages from normal mice with immune spleen cells and specific antigen induced tumoricidal macrophages. In vitro activation of normal macrophages also occurred with supernatants for antigen-stimulated immune spleen cell cultures (lymphokines). Tumoricidal activity was evident after 3 hours of culture in lymphokines, was optimal at 8-12 hours, and disappeared at 20 hours. Also, the capacity of normal macrophages to be activated by lymphokines progressively decreased with time in culture and was absent at 20 hours. The loss of capacity for activation was not due to cell death. After PPD injection intraperitoneally to BCG immune mice, cytotoxic cells appeared and then disappeared within 20 hours of antigen clearance. The loss of cytotoxic cells reflected the short-lived nature of both the cytotoxic capacity of activated macrophages and the capacity of precursor cells to be activated by lymphokines.

Several functional properties distinguished activated tumoricidal macrophages from normal macrophages or macrophages elicited by irritants. These included increased response to chemotactic stimuli and release of a soluble monokine that was mitogenic for T-lymphocytes (LAF). BCG-activated macrophages released 3-5 times more LAF than equal numbers of normal macrophages. Macrophages from mice treated with thioglycollate or mineral oil were not cytotoxic and did not produce increased LAF.

It was shown last year that chemotaxis of mouse peritoneal macrophages required serum in the cell suspension and that the serum effect was mediated by a single macromolecule with a M.W. of about 100,00. MSP (macrophage stimulating protein) has been further purified in a series of steps that include G-200 Sephadex and DEAE chromatography, isoelectric focusing (P.I. = 7.0), absorption with Cowan strain S. aureus to remove traces of IgG, and polyacrylamide gel electrophoresis. MSP activity per mg protein was increased about 400-fold. The potent MSP activity in whole serum was mediated by less than 5 ug/ml of the protein.

B. Lymphotoxin. The term lymphotoxin has been applied to the factors in supernatant fluids of stimulated lymphocyte cultures that destroy tumor cell lines, although cell counting assays for lymphotoxin may detect factors that inhibit proliferation as well as those that kill. An assay of cell killing is release of thymidine from prelabelled target cells; it has been used in this laboratory for isolation and purification of mouse lymphotoxin. Further study of the conditions for LT assay have shown (1) the sensitivity of cells to LT action was greatly increased by tritiated thymidine labelling. This was due to the radiation, not to thymidine. (2) Addition of DNA-ase, as late as 15 min before the end of the 72 hour assay, doubled tritium release. After the death of cells, release of nuclear thymidine was not inevitable and could be increased by manipulation of culture conditions. (3) LT was equilibrated with target cells for 24 hours and then removed; there was no thymidine release at 24 hours. The chain of events leading to cell death and thymidine release continued despite removal of LT. Counts released at 72 hours were at least 60% of the counts released after 72 hours with LT. (4) There was no detectable absorption of LT by target cells, as determined by titration of LT remaining in fluids after equilibration with target cells. This suggested weak binding to target cell receptors.

C. Basophils in Inflammation. The composition of the leukocyte infiltrate in delayed cutaneous hypersensitivity lesions of guinea pigs is determined by the mode of immunization. In the classical tuberculin reaction, basophils are rare; whereas the response to hemocyanin in animals primed with hemocyanin in saline is a lesion rich in basophils. There are more basophils in one such lesion than in the total circulating blood volume of the guinea pig. Therefore antigenic challenge must initiate a remarkable sequence of events that signal the release and perhaps maturation of basophils from bone marrow or as yet unknown sites.

Human peripheral blood basophils migrate toward chemotactic factors, including complement C5a and substances in culture fluids of

stimulated lymphocytes (LDCF). Addition of culture fluids to basophil suspensions increased the chemotaxis of basophils to C5a. The activity in the culture fluids was called basophil chemotaxis augmentation factor (BCAF). It is a macromolecule with a molecular weight of about 30,000. It was separated from LDCF, which has a M.W. of about 12,000. Experiments with the purified fractions showed that BCAF increased basophil chemotaxis to LDCF as well as C5a.

Among the interesting aspects of basophil cell biology are (1) only a fraction of the blood basophils migrate toward chemotactic factors, but the size of this fraction can be increased rapidly by BCAF. Other evidence for basophil subpopulations is the inhibition of migration by histamine, affecting all but a fraction that is not inhibited by even high concentrations of the drug. (2) Addition of C5a to basophils, followed by washing, inhibited chemotaxis to C5a but did not affect chemotaxis to LDCF. Similar results were obtained by addition of LDCF to basophils. This shows the specificity of receptors for chemotactic factors. (3) Histamine inhibits histamine release from basophils. The chemotactic response of basophils to C5a was inhibited by low concentrations (10^{-8}M) of histamine. It would be of interest to know whether this constitutes a negative feedback control of basophil accumulation at sites of inflammation. The effect of histamine was abolished by prior addition of H2-receptor inhibitors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08525-01 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Primary Autochthonous Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. J. Rapp Chief, Laboratory of Immunobiology LIB NCI

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Immunobiology, Immunology Program

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals with experimentally induced or naturally occurring primary autochthonous cancer are being studied as guides to the immunotherapy of human cancer. It is hoped that these guides will provide information more relevant to the treatment of human cancer than that derived from studies with transplanted tumors. Cattle with ocular squamous cell carcinoma ("cancer eye") have been treated successfully by the injection of BCG cell wall vaccine directly into the tumor.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The primary objective of this project is to test a variety of immunologic cancer therapies in several species with primary cancer in the hope of providing guides to the immunotherapy of human cancer. The therapeutic methods tested will be those that have been successful in animal models consisting of syngeneic tumor transplants.

Methods Employed: Hereford cattle were examined at feed lots for evidence of "cancer eye." Suspected animals were purchased and transported to Colorado State University. Diagnosis was established by microscopic examination of stained smears from lesions. Animals with papillomas were not entered into the treatment protocol. Cattle with histologically confirmed carcinoma were divided into three groups. Twenty-four animals in group I received a single treatment consisting of multiple injections of BCG cell wall vaccine into the tumor. Nine animals in group II received the ingredients of the vaccine deliberately compounded improperly. Nine animals in group III received no treatment.

Major Findings: Of the 24 animals treated with proper vaccine the disease was and has remained arrested in 8 (33%), 2 (8%) have tumors that are in the process of regression and 7 (29%) have had complete regression of tumor. Some treated animals have been free of tumor for more than two years. The remaining 7 animals as well as all 18 control animals have progressive disease or have been killed because the disease advanced to a stage that caused pain. At autopsy most animals with advanced disease were found to have metastases.

Significance to Biomedical Research and the Program of the Institute: Clinical cancer immunotherapy has been proceeding with minimal guidance from the experimental laboratory. The lack of fundamental information for the design of clinical protocols has necessitated an empirical rather than a rational approach to the immunotherapy of human cancer. Among current necessary practices in cancer immunotherapy are 1) The use of immunotherapy in advanced malignancy after other forms of treatment, 2) Multiple treatments with immunologically active agents that are safe if given once but potentially lethal when given more than once, 3) Administration of agents by routes that are not curative in animal models, 4) Modification of clinical trial protocols while a study is in progress and 5) The use of historical controls in clinical trials rather than randomization. Practices of these kinds have generated information that may discourage appropriate application of immunologic principles to the control of human cancer. One possible factor contributing to this situation is the lack of suitable animal studies to provide the information necessary to design rational clinical protocols. Bovine ocular squamous cell carcinoma provides a potential guide lacking the undesirable aspects of currently available experimental models and perhaps the means to establish useful approaches to the immunotherapy of human cancer. Among the similarities to human cancer and advantages of the bovine model are: 1) The size of the animal

makes it possible to take large tissue samples and quantities of blood for study without adverse effects on the animal. 2) The animals are long lived, thus permitting an adequate follow up. 3) Surgical techniques and drug dosages are similar to those used in humans. 4) Potentially metastatic, primary autochthonous carcinomas that develop and grow slowly are the major cause of human cancer deaths. 5) Exfoliative cytology can be done without surgery. 6) It may be possible to delineate the factors that contribute to the failure of the immune system to control the development of naturally occurring cancer. 7) Thousands of animals with "cancer eye" are available in the United States as well as other countries where large numbers of cattle are raised. 8) Studies with "cancer eye" are reasonable because the expense during carcinogenesis is borne by the raiser and not the investigator and the cost of housing each animal compares favorably with smaller, laboratory housed animals.

Proposed Course of Project: While in this study a single intralesional injection of BCG either arrested or caused regression of most of the bovine ocular squamous cell carcinomas, it is not known whether the disease had spread beyond the primary site at the time of treatment. Experiments are now in progress to compare the therapeutic efficacy of local surgery and intralesional BCG for "cancer eye." The results of these experiments will help to answer the question of whether, as in guinea pigs, intralesional BCG also eradicates microscopically detectable regional lymph node metastases. Additional studies of "cancer eye" are needed to determine whether the outcome of therapy with BCG cell wall vaccine is influenced by the presence of metastases, the size and location of tumors and the condition of the animals.

Publications

Kleinschuster, S.J., Rapp, H.J., Leuker, D.C., and Kainer, R.A.: Regression of bovine ocular carcinoma by treatment with a Mycobacterial vaccine. J. Natl. Cancer Inst. 58: 1807-1814, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08526-01 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Immunologic Approaches to the Treatment of Animals with Stage I Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunology	LIB NCI
	B. Zbar	Senior Investigator	LIB NCI
OTHER:	J. Hunter	Expert	LIB NCI
	G. Canti	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Cellular Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by methods involving immunostimulation. The areas of current interest are 1) the determination of conditions required for immunologic eradication of lymph node metastases and 2) the study of the cellular and molecular basis of eradication of tumor cells within lymph nodes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The primary objective of this project is to define the conditions and methods best suited to the prevention and treatment of cancer by procedures involving immunostimulation. This objective is based on the finding that tumors contain unique antigens capable of eliciting the formation of specific humoral antibodies and of causing the appearance of cells which react specifically with the tumor antigens.

Methods Employed: Transplantable ascites tumors were derived from primary hepatomas induced in strain-2 male guinea pigs by feeding of diethylnitrosamine. These ascites tumors are maintained by periodic intraperitoneal transplantation. Most of the recent studies have been carried out using the hepatoma line-10 which when injected into the skin, produces a progressively growing papule and local metastases within a few days.

Tumor therapy with immunostimulants (BCG, BCG cell wall vaccines, and Glucan) was carried out by the intratumor or intradermal injection of immunostimulants into unimmunized guinea pigs when established intradermal tumors and regional lymph node metastases were present.

A model for treatment of animals with bilateral axillary lymph node metastases was used in which line-10 tumor cells were injected intradermally over the thoracic spine. In some experiments, lymph nodes were excised, disaggregated, and the cell suspension transplanted by the intraperitoneal route.

Tumor cells were injected intradermally over the flank, axilla, shoulder or foot to determine the distribution of lymph node metastases. Tumor cells were injected intradermally over the flank or axilla to determine the effects of regional BCG treatment on microscopic lymph node metastases.

Major Findings: Development of an animal model for bilateral lymph node metastases. (1) Intradermal injection of tumor cells over the thoracic spine led to the development of bilateral SDA lymph node metastases. (2) Tumor cells were present in both SDA lymph nodes of guinea pigs by 7 days after tumor cell injection. (3) The presence of tumor cells in both SDA lymph nodes on day 7 was demonstrated by noting the development of metastatic disease following local tumor excision and by transplantation of disaggregated lymph nodes to the peritoneal cavity of weanling guinea pigs. Of 30 SDA lymph nodes transplanted, 28 recipients developed ascites tumors. (4) Intralesional injection of BCG into midline intradermal tumors led to eradication of the dermal tumor and the bilateral microscopic lymph node metastases. (5) Intradermal injection of BCG into the right and left flanks prior to local excition was effective in destroying the bilateral microscopic malignant disease. Local surgery was required for these treatments to be effective. (6) Intradermal injection of BCG into the left flank prior to local excision was effective in destroying metastases in the left SDA lymph node but failed to influence the growth of metastases in the right SDA lymph node.

Conditions required for eradication of microscopic malignant lymph node metastases. (1) Tumor cells injected into the skin of the flank, lateral aspect of the shoulder, and medial and lateral aspect of the front foot metastasized to the SDA lymph node. (2) Tumor cells injected into the skin of the axilla metastasized to the PA lymph node. (3) Injection of BCG into the flank adjacent to the flank dermal tumor prior to local surgery led to destruction of metastases in the SDA lymph node; intradermal injection of BCG into the axilla prior to excision of the flank tumor failed to inhibit growth of tumor in the SDA lymph node. (4) Intradermal injection of BCG into the axilla adjacent to a dermal axillary tumor prior to local surgery led to destruction of metastases in the PA node; intradermal injection of BCG into the flank prior to excision of the flank tumor failed to influence growth of tumor in the PA node.

Evaluation of antitumor activity of glucan. (1) Intralesional and intravenous injections of dextrose and water suspensions of glucan did not cure animals with the line-10 hepatocarcinoma. (2) Mixtures containing glucan, P3 and mineral oil when injected intralesionally did not cure animals with line-10 tumor.

Significance to Biomedical Research and the Program of the Institute. The line-10 tumor of strain-2 guinea pigs has been useful in (1) the evaluation of potential antitumor activity of compounds. (2) The study of biologic effects of immunostimulants in animals at different stages of malignant disease and in (3) devising routes and schedules of immunologic treatments effective against lymph node metastases. The results of current work indicate that in this model glucan has no significant antitumor activity and suggests that clinical testing with glucan should be deemphasized. The success of intralesional BCG treatment in animals with bilateral microscopic lymph node metastases suggests that intralesional treatment may be of value in patients with microscopic malignant disease in several lymph nodes. Successful treatment of lymph node metastases requires drainage of adjuvant into all lymph nodes which contain tumor cells. Experiments with dermal and axillary tumors indicate that there are critical anatomic requirements which determine success of treatment of microscopic lymph node metastases by regional administration of adjuvants. Lymph node transplantation may prove to be a successful method for studying BCG mediated destruction of lymph node metastases.

Proposed Course of Project. Additional experiments will be performed on the effects of unilateral BCG treatment in animals with bilateral lymph node metastases. Tumor cells will be injected to produce microscopic metastases in PA and SDA lymph nodes. Tests will determine whether destruction of modest numbers of tumor cells in the SDA node will lead to destruction of modest numbers of tumor cells in the adjacent PA lymph node. Occasional experiments suggested that administration of adjuvant after surgery eradicated microscopic lymph node metastases. Living BCG, BCG cell walls attached to oil droplets and a combination of BCG cell wall skeleton, P3 and endotoxin will be evaluated for ability to reproducibly eliminate metastases when administered after excision of the dermal tumor.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08527-01 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Immunologic Approaches to Treatment of Animals with Stage II Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J. Hunter	Expert,	LIB NCI
OTHER:	B. Zbar	Senior Investigator	LIB NCI
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by methods involving immunostimulation. The areas of current interest are: 1) the development of immunologic methods effective in the treatment of animals with stage II malignant disease and 2) evaluation of tumor immunity in tumor bearing animals and in animals cured of malignant disease by surgery.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The primary objective of this project is to determine the role of "immunity" as manifested by intradermal tumor challenge on the antitumor effect of immunoadjuvant therapy. This objective is based on our recent observation that removal of line-10 tumor by radical surgery 8 days after tumor implantation did not lead to development of resistance to contralateral tumor challenge, whereas radical surgery 16 days after tumor challenge led to resistance to contralateral challenge. Previous experiments on intralesional therapy were generally conducted at a time when the host was not "immune" to the tumor whereas treatment of stage II disease is initiated at a time when the host is "immune".

Formerly, it has been considered that immunoadjuvant therapy led to the development of tumor immunity and that the "immunity" was associated with the destruction of metastatic tumor in the draining nodes. The fact that immunity develops in the absence of adjuvant therapy following surgery at 16 days suggests that it is possible that resistance to intradermal challenge is not related to the mechanism of BCG antitumor activity.

Methods Employed: A transplantable syngeneic hepatocellular carcinoma, designated line-10, in transplant generations 8-18 was used. This diethylnitrosamine-induced tumor had been converted to an ascites form and is passed ip in weanling strain-2 male guinea pigs. Inoculation of 10^6 line-10 tumor cells ID leads to progressive tumor growth and metastasis to the regional axillary lymph node by 7 days after tumor implantation.

Intradermal tumor challenge is carried out by injecting 10^6-10^4 cells ID into the skin of the contralateral flank. Challenge sites were examined weekly for 3 consecutive weeks after injection for evidence of tumor growth.

Surgery is carried out utilizing ether and pentobarbital anesthesia. The dermal tumor is excised with a 1cm skin margin and the draining lymph node chains are dissected out.

Intralesional BCG therapy is carried out by injecting the tumor with 0.4 ml of an oil saline emulsion containing 850 ug of BCG cell wall.

A model for treatment of clinically detectable lymph node metastases by radical lymph node dissection and BCG therapy was used in which one million line-10 cells were injected intradermally into strain-2 guinea pigs. When palpable lymph node metastases were detected, BCG was injected into the tumor and 7 days later the tumor was excised and regional lymph node dissection performed. Palpable metastases were first detectable when the nodes were 6-7 mm in diameter, approximately 21 days after tumor implantation.

Major Findings: Animal model for stage II malignant disease. (1) Radical surgery was effective in curing about 50% of guinea pigs with intradermal tumors and palpable superficial distal axillary (SDA) lymph nodes. (2) Suc-

cessful surgery required removal of the dermal tumor, SDA lymph node and fat pad and the proximal axillary (PA) lymph nodes. (3) Tumor transplantation immunity was detectable in approximately 50% of the animals successfully treated by surgery. (4) Intralesional injection of BCG prior to surgery did not increase the incidence of tumor free animals. (5) Modified radical surgery failed to cure animals with intradermal tumors and palpable SDA lymph nodes. Modified radical surgery consisted of excision of the dermal tumor and the SDA lymph node and fat pad. Animals died of progressive tumor growth in the PA lymph node. (6) Intralesional BCG treatment prior to modified radical surgery failed to cure animals with stage II disease. (7) Several immunologic modalities were used in an effort to eradicate metastatic disease remaining in the PA lymph node after modified radical surgery. These supplementary treatments included transfusion of lymphoid cells from immune donors, injection of BCG adjacent to the dermal tumor, and injection of BCG into the skin of the axilla. None of these treatments succeded in eradicating metastases in the PA lymph node. (8) Treatment of the Stage II malignant disease by intralesional injection of 10 mg of BCG cell wall in oil (the maximal amount we are currently capable of giving) had no benefical therapeutic effects. (9) Treatment with adriamycin (2 mg/Kg) intratumor followed one day later by 800 ug of BCG cell wall I.T. did not produce any benefical therapeutic effects.

Tumor immunity in animals cured of tumor by radical surgery. (1) Resistance to intradermal challenge with 10^6 L-10 cells was not detectable if the challenge was given on the day of surgery but approximately 50% of surgically cured animals resisted challenge by 7 days after surgery. This "immunity" was detectable for at least 180 days. Surgery of normal animals did not lead to resistance to tumor cell challenge. (2) Concomitant immunity to 10^6 tumor cell challenge was not detectable at any time during the growth of 10^5 or 10^6 implaned tumor cells. (3) No resistance to tumor challenge was evident when the tumor mass was reduced early on by excising the primary tumor at day 7 and then excising the draining nodes (with metastatic tumor) at 21 days after tumor implantation. (4) The presence of tumor immunity induced by vaccination with tumor cells and BCG cell wall emulsified in oil on day 3 post tumor implantation did not interfere with the capacity of intralesionally injected BCG cell wall emulsion at day 10 to bring about the destruction of the primary tumor and the node metastases.

Significance to Biomedical Research and the Program of the Institute. Human therapy with intralesional BCG has been most successful in treatment of stage I dermal tumors and it has been reported to prevent recurrence after surgery of stage I squamous cell carcinoma of the lung. Therapy of stage II or more advanced disease has not been strikingly effective. The relationship between systemic tumor immunity and the more local effects of BCG injection may elucidate the difficulties.

Proposed Course of Project. To continue to conduct experiments to determine if concomitant immunity can be detected when lower doses of tumor challenge are given. To further study the effect of tumor burden on immunity by injecting lower initial tumor inocula. Efforts will be made to treat stage

II residual disease by vaccination with BCG and tumor cells in an effort to stimulate secondary immune responses.

Publications

Bast, R. C., Jr. Bast, B. S. and Rapp, H. J.: Critical review of previously reported animal studies of tumor immunotherapy with nonspecific immunostimulants. Ann. N. Y. Acad. Sci. 277: 60-93, 1976.

Rapp, H. J.: Immunotherapy of experimental cancer with BCG. In Crowell, R. L. Friedman, H. and Prier, J. E. (Eds.): Tumor Virus Infections and Immunity. Baltimore, University Park Press, 1976, pp. 261-264.

Rosenberg, S. A. and Rapp, H. J.: Intralesional immunotherapy of melanoma with BCG. Med. Clin. North Am. 60: 419-430, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08528-01 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
	A. Altman	Visiting Fellow	LIB NCI
OTHER:	M. S. Meltzer	Surgeon	LIB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Immunobiology, Immunology Program

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to elucidate the mechanism of the immunologic rejection of tumor grafts and to determine the mechanism of tumor suppression following immunostimulation with BCG. Current areas of investigation are (1) induction of syngeneic tumor immunity in vitro and (2) the relationship between tumor immunogenicity and the induction of suppressor cells in tumor bearing mice.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The objectives of this project are to develop a system for the primary in vitro immunization of guinea pig lymphocytes against syngeneic tumors, to study whether the pattern of induction of suppressor cells is related to the immunogenicity of tumors and to determine whether suppressor cells have a biological role in the regulation of the host-tumor relationship.

Methods Employed: In vitro sensitization. (1) Hepatomas 13C and 13F, induced by feeding diethylnitrosamine, and fibrosarcoma 1303 induced by injection of 3 methylcholanthrene were kept frozen, and passed in vivo by sc injection of enzymatically digested tumor cells. (2) Primary cultures of the above tumors were established, and maintained by serial passage in cell culture. (3) Single cell suspensions of normal unimmunized, strain-13 guinea pig lymph nodes were prepared and the lymph node cells (LNC) were cultured with mitomycin C treated tumor cells for 4-5 days in Dulbecco's modified Eagle's medium supplemented with 5% serum (guinea pig or fetal calf) and 5 x 10^5 M. 2 mercaptoethanol (2-ME). (4) The effector activity of the cultured lymphocytes was determined by incubation with fresh cultures of tumor target cells. Following such cocultivation the amount of target cell damage was measured by labelling the surviving target cells with a radioactive label (^{51}Cr).

Suppressor Cells. (1) The fibrosarcomas 1023 and 1063, induced in C3H/HeNIcr male mice by SC implantation of methylcholenthrene were kept frozen and passaged by SC trocar inoculation of tumor tissue in immunosuppressed C3H/HeN male mice. (2) Immunogenicity of the two tumors studied was evaluated by the following techniques; a. Implantation and excision of the tumor and subsequent challenge with tumor cells. b. Intralesional injection of live BCG organisms into established tumors. c. Vaccination with mixtures of tumor cells and BCG. (3) Mice were injected with 10^6 1023 or 1063 tumor cells ID. At various times thereafter, spleens of tumor bearing (TB) mice were removed and single cell suspensions prepared. (4) Increasing numbers of TB or normal control spleen cells were added to a constant number of normal spleen cells and the proliferative response of these cultures to the mitogen concanavalin A was measured by ^3H-thymidine uptake. (5) Various mouse spleen cell populations were purified and defined by treatment with anti-θ serum and complement, passage on nylon fiber columns, incubation with carbonyl-iron and elution over magnet, adherence to plastic etc.

Major Findings: In vitro sensitization. (1) LNC cultured with syngeneic tumor cells became functionally sensitized in vitro as evidenced by ability to damage target cells. (2) The terminal labelling assay for the measurement of target cell damage was found to be a reliable and quantitative assay. (3) Lymphocytes cultured in medium containing fetal calf serum, even in the absence of tumor cells, were found to be nonspecifically cytotoxic to various types of tumor cells and normal fibroblasts. (4) This nonspecific cytotoxicity ("natural killer" cell activity?) could be reduced

or eliminated by passing the Lnc through nylon fiber columns prior to their sensitization in vitro, or by eliminating 2-ME from the tissue culture medium. (5) In addition, we found that lymphocytes could be sensitized efficiently in vitro in the presence of fresh (but not stored) syngeneic guinea pig serum. Under such conditions, the nonspecific "natural killer" activity was eliminated, and a more restricted pattern of cytotoxicity was observed. Thus, lymphocytes cultured in medium alone or with normal kidney fibroblasts and with GPS did not become cytotoxic, whereas LNC cultured with hepatoma cells became cytotoxic to hepatoma cells and to a lesser extent to fibrosarcoma cells but not to normal fibroblasts.

Suppressor cells. (1) In accordance with previous findings in this laboratory it was found that the 1023 fibrosarcoma is strongly immunogenic whereas the 1063 tumor is only weakly immunogenic. (2) The proliferative response of TB spleen cells to various concentrations of concanavalin A was depressed over a wide range of cell densities in vitro. (3) This depressed reactivity was due to the presence of suppressor cells, as evidenced by the ability of TB spleen cells to suppress the proliferative response of normal spleen cells. (4) The 1063 tumor induced an early peak of suppressor cells detectable between 3 and 7 days after tumor inoculation. Such an early peak was not detectable in the 1023 tumor system. (5) Suppressor cells were detected in the spleens of 1023 or 1063 TB mice between 2 and 3 weeks following tumor inoculation and persisted till the death of the mice. (6) The suppressor cells found in the spleens three weeks after tumor inoculation and later are non T, phagocytic and adherent to plastic or nylon fibers, and thus, appear to be macrophages.

Significance to Biomedical Research and the Program of the Institute. Sensitization of lymphocytes to syngeneic tumor antigens in vitro may overcome immunoregulatory homeostatic mechanisms which prevent efficient induction and/or expression of tumor specific immunity in vivo, and may lead to the establishment of a useful therapeutic approach. Elucidation of the relationship between tumor immunogenicity and suppressor cell induction is important for the understanding of the possible biological role of suppressor mechanisms in the tumor-host relationship.

Proposed Course of Project: (1) To study the in vivo anti-tumor activity of in vitro sensitized lymphocytes. (2) To study and elucidate the nature of restricted cross-reactivity observed in this system in the cytotoxicity assay. (3) To try and augment the efficiency of in vitro sensitization by various manipulations in the cultures. (4) To study whether the relationship between tumor immunogenicity and suppressor cell induction, as found in this study, exists in other tumor systems. (5) To measure suppressor cell activity in assays of specific tumor immunity, in vitro as well as in vivo.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08529-01 LIB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunotherapy with Defined Bacterial Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. Rapp	Chief, Laboratory of Immunobiology	LIB NCI
	E. Yarkoni	Visiting Fellow	LIB NCI
OTHER:	J. Hunter	Expert	LIB NCI
	B. Zbar	Senior Investigator	LIB NCI
	L. Ruco	Visiting Fellow	LIB NCI
	M. Meltzer	Surgeon	LIB NCI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Components of mycobacteria injected in oil/water emulsions into syngeneic tumors growing in the skin of guinea pigs or mice caused the tumors to regress. Animals cured of their tumors were resistant to rechallenge with the same tumor.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The main objective of this project is to isolate and define mycobacterial components with maximal antitumor effects and minimal toxicity.

Methods Employed: Malignant hepatomas were induced in strain-2 guinea pigs by the feeding of diethylnitrosamine. Ascites variants of the tumors were maintained by serial intraperitoneal passage.

Fibrosarcomas arose in the subcutaneous tissues of a C3H/HeNIcr male mice after implantation of paraffin pellets containing 1% 3-methylcholanthrene. The tumors were maintained by serial trocar passage in syngeneic immunodepressed mice.

Mineral oil/water emulsions of mycobacterial vaccines were prepared by ultrasonication or grinding. The following mycobacterial components were investigated: Mycobacterial whole cells or cell walls and trehalose-6,6'-dimycolate (cord factor).

Emulsified mycobacterial vaccines were infiltrated into the growing tumors. The tumors were subsequently observed for progression or regression.

Mycobacterial vaccines were injected intravenously into normal animals and their weight and deaths were recorded.

Mycobacterial vaccines in combination with lyophilized hepatoma cells were injected once intradermally into guinea pigs. The animals were challenged with viable tumor cells 0, 14, or 21 days thereafter and were observed for tumor growth or rejection.

Major Findings: (1) Oil in water emulsions containing either mycobacterial cell walls or killed whole cells attached to the oil droplets were prepared by ultrasonication or by grinding. The immunotherapeutic potency of the ultrasonically prepared vaccines was at least as great as that of similar vaccines prepared by emulsification in a tissue grinder. (2) Emulsified BCG cell walls were compared to emulsions of trehalose-6,6'-dimycolate (TDM) in admixture with endotoxin (ET) for their ability to cause regression of a transplanted guinea pig hepatoma. On a weight basis cell walls were at least as active as ET-TDM. Toxicity of ET and of ET-TDM were greater than that of cell walls as judged by weight loss after intravenous administration of emulsions to normal guinea pigs. (3) Living BCG, killed Mycobacterium tuberculosis cells or BCG cell walls augmented the immunogenicity of lyophilized syngeneic ascites hepatoma (line-10) cells of strain-2 guinea pigs. Effective vaccines contained living BCG and lyophilized line-10 cells, or mycobacterial cells or cell walls attached to oil droplets and lyophilized line-10 cells. Protection against the challenge tumor was evident 14 or 21 days after one administration of either vaccine. (4) Injection of emulsified trehalose-6,6'-dimycolate (TDM) into transplants of an established, syngeneic murine fibrosarcoma induced complete regression of tumor in a number of

animals. The number of animals in which tumors regressed completely depended on the amount of oil in the emulsion. The antitumor activity of the TDM emulsion containing the highest concentration of oil was equivalent to that of living BCG. (5) The increased endotoxin lethality in mice pretreated with BCG intravenously, was not observed in mice pretreated (iv) with trehalose-6,6'-dimycolate instead of BCG.

Significance to Biomedical Research and the Program of the Institute: Immunotherapy with BCG cell walls and trehalose-6,6'-dimycolate with or without endotoxin has proven to be effective treatment when the tumor mass is small and microscopic metastases are present. There are clinical situations in which therapy with mycobacterial vaccines may be a practical and desirable approach. Killed BCG cell vaccines with or without trehalose-6,6'-dimycolate have been tried successfully in preliminary studies of the treatment of human skin cancer.

Proposed Course of Project: Experiments designed to determine the requirements for successful immunotherapy will be continued. Emphasis will be placed on the isolation and definition of additional components of mycobacteria which have antitumor effects. Efforts will be made to develop vaccines which cause regression of dermal tumors and palpable lymph node metastases.

Publications

Rapp, H. J., Yarkoni, E. Ruco, L. and Hunter, J. T.: Immunotherapy of a guinea pig hepatoma with vaccines containing mycobacterial fractions: comparison of cell walls and trehalose-6,6'-dimycolate. Cancer Immunol. Immunother, in press.

Yarkoni, E., Meltzer, M. S. and Rapp, H. J.: Failure of trehalose-6,6'-dimycolate (P3 or cord factor) to enhance endotoxin lethality in mice. Infect. Immun. 14: 1375-1377, 1976.

Yarkoni, E., Meltzer, M. S. and Rapp, H. J.: Tumor regression after intralesional injection of emulsified trehalose-6,6'-dimycolate (cord factor): Efficacy increases with oil concentration. Int. J. Cancer, in press.

Yarkoni, E., Rapp, H. J. and Zbar, B.: Immunotherapy of a guinea pig hepatoma with ultrasonically prepared mycobacterial vaccines. Cancer Immunol. Immunother., in press.

Yarkoni, E., Zbar, B. and Rapp, H. J.: Resistance to tumor growth in guinea pigs immunized with lyophilized tumor cells. Cancer Immunol. Immunother., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08550-03 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modification of Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. I. Schlager	Staff Fellow	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	M. D. P. Boyle	Expert	LIB NCI
	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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Humoral Immunity Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pretreatment of tumor cells with chemotherapeutic agents, metabolic inhibitors, enzymes or hormones modifies the susceptibility of the cells to killing by antibody and complement. The purpose of this investigation is to determine the attributes of the cell which are modified following treatment with the agents.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To study the mechanisms whereby enzymes and metabolic inhibitors increase the sensitivity of tumor cells to immune attack; 2) to study mechanisms whereby hormones decrease sensitivity of cells to immune attack; 3) to determine the physical and chemical processes and cell products which may be modified following such treatment; 4) to determine the processes which may be modified following treatment with antibody and complement.

Methods Employed: Antibody is quantitated by the complement fixation and transfer test and immune cytolysis. Sensitivity of cells to attack is measured by uptake of trypan blue and/or release of ^{125}IUdR. Incorporation of radio-labelled precursors of DNA, RNA, protein, complex carbohydrate, glycoproteins, glycolipids and lipids are being used to measure general metabolic properties of the cells. Thin layer chromatography is being utilized to analyze and identify the particular lipid moieties associated with the cells.

Major Findings: Glucocorticoid and polypeptide hormones render line-1 and line-10 guinea pig hepatoma cells resistant to killing by antibody and complement. The effect is dose and temperature dependent and reverses even in the continued presence of cell-bound hormone. Hormone-treated reversed cells can be rendered resistant to killing after incubation with a different, but not with the same, hormone. In addition, hormone pretreatment of line-10 cells reduces their sensitivity to killing following treatment with proteolytic enzymes. The metabolic requirements for hormones to be effective in rendering tumor cells resistant to antibody-complement mediated killing are that cells have at least RNA and protein synthesis intact. There is no absolute requirement for DNA synthesis.

The ability of line-1 and line-10 tumor cells to resist humoral immune attack depends on their ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate. Cells treated for short periods or with low doses of metabolic inhibitors or chemotherapeutic agents are not rendered susceptible to killing and are not inhibited in their lipid synthesis; these cells are inhibited in their synthesis of DNA, RNA, protein, and complex carbohydrate. Prolonged incubation of the cells with high concentrations of drugs renders the cells sensitive to killing and inhibits their ability for complex lipid synthesis. Both of these effects reverse within 4 hours of reculture without drug; the cells do not regain their ability to synthesize DNA, RNA, protein, or complex carbohydrate.

Tumor cells irradiated with 6000 or 9000 R are rendered susceptible to antibody-complement mediated killing 6 hours post-irradiation and remain sensitive 16 hours post-irradiation. Cells heated 30 min at 43, 44 or 45°C do not become susceptible. The sensitivity or resistance of the tumor cells following either physical treatment correlated with their inability or ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate.

Tumor cells treated with antibody and GPC demonstrate increased synthesis of complex lipids. No such increase is observed in cells treated with antibody and HuC.

Significance to Biomedical Research and the Program of the Institute: Modification of tumor cells by metabolic inhibitors, enzymes, hormones, and chemotherapeutic agents furnishes a new tool to study the interaction of tumor cells with the immune defense mechanisms of the host.

Proposed Course of Project: Radioisotope incorporation studies will be continued as probes to determine the cellular functions which are modified by treatment with enzymes, inhibitors or hormones. The chemical attributes of normal cells, tumor cells and treated tumor cells will be studied. Work will continue on the effect of enzymes, hormones and inhibitors of macromolecular synthesis on the metabolic function of the tumor cells.

Publications

Schlager, S. I., Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Effect of inhibiting DNA, RNA, and protein synthesis of tumor cells on their susceptibility to killing by antibody and complement. Cancer Res. 37: 1432-1437, 1977.

Schlager, S. I. and Ohanian, S. H.: Correlation between lipid synthesis in tumor cells and their sensitivity to humoral immune attack. Science, in press.

Schlager, S. I., Ohanian, S. H. and Borsos, T.: Inhibition of antibody-complement mediated killing of tumor cells by hormones. Cancer Res. 36: 3672-3677, 1976.

Schlager, S, I., Ohanian, S. H. and Borsos, T.: Kinetics of hormone-induced tumor cell resistance to killing by antibody and complement. Cancer Res. 37: 765-770, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08551-02 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Induction of Tumor Immunity by Chemotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	S. I. Schlager	Staff Fellow	LIB NCI
	E. Yarkoni	Visiting Fellow	LIB NCI
	B. Zbar	Senior Investigator	LIB NCI
	J. T. Hunter	Expert	LIB NCI

COOPERATING UNITS (if any)

None

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Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected chemotherapeutic agents injected into tumors growing in vivo cause the tumor to regress. Animals cured of their tumors are resistant to rechallenge with the same tumor. The purpose of this work is to analyze the mechanisms of intralesional chemotherapy and include histological techniques, and methods for analyzing humoral and cellular mediated immune mechanisms.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To cure tumors and induce tumor immunity by direct treatment of established tumors with chemotherapeutic agents; 2) to study the development of cellular and humoral immunity in cured animals.

Methods Employed: Inhibition of tumor growth in animals cured of tumors by intratumoral chemotherapy is used to measure the development of tumor immunity. Intradermal injections of the ascites form of line-10 tumor cells is used to determine malignancy. Immunological techniques employed to measure humoral and cellular immunity include C fixation, antibody-complement mediated killing and cell mediated cytotoxicity.

Major Findings: Tumor cell growth in the skin of guinea pigs can be inhibited by injection of melphalan, adriamycin, mitomycin C or BCNU into the 7-day-old developing tumor. At the time chemotherapy was started, most animals had metastases in the draining lymph node. The effect is dependent upon dose of inhibitors. Multiple intralesional injection was as effective as a single injection. 5 FU, DTIC, methotrexate and 6 MP were not effective. None of the chemotherapeutic agents was effective if administered intraperitoneally. Animals cured of their tumors were tested for immunity by rechallenge with line-10 or line-1 tumor cells. All animals were immune to rechallenge with 10^6 line-10 cells but not line-1 cells.

Drugs which were effective in curing animals of 7-day-old tumors were not effective in animals having 6-7 mm lymph nodes (tumors 21-35 days old) or in animals with 14-day-old tumors. In addition, adriamycin and BCNU were toxic for the animals.

Excision of primary tumor when lymph nodes reached 6-7 mm followed by intradermal chemotherapy with adriamycin, BCNU or melphalan is not an effective means to cure animals of the residual metastases.

Histological examination of 7-day-old tumor injected with adriamycin or DTIC shows massive necrosis of tumor and surrounding tissue. The pigment of adriamycin stained tumor cells and tissue and persisted for weeks. Compared to tumor sites injected with DTIC a lesser number of lymphocytes and histiocytes were noted in the tumor treated with adriamycin. Intralesional injection with adriamycin prevented growth of tumor cells in the draining lymph nodes within 7 days. DTIC treatment was not effective in preventing tumor growth in the draining lymph nodes.

Significance to Biomedical Research and the Program of the Institute: These studies in a syngeneic tumor animal model system are designed for analyzing the mechanism of intralesional chemotherapy. The development of this system should make it possible to better utilize intratumoral therapy of human cancer.

Proposed Course of Project: Further studies on the effects of intratumoral chemotherapy into tumors growing for longer than 7 days. Histology of the tumor sites at various times after injection of chemotherapeutic agents will be studied. Studies will be performed to determine the effect of multiple small injections of drugs into tumors growing for longer than 7 days. Techniques of humoral and cellular immunity to the tumor will be employed to more critically define the immune status of the treated animals.

Publications

Borsos, T., Bast, R. C., Jr., Ohanian, S. H., Segerling, M., Zbar, B. and Rapp, H. J.: Induction of tumor immunity by intratumoral chemotherapy. Ann. N. Y. Acad. Sci. 276: 565-572, 1976.

Ohanian, S. H., Yarkoni, E., Hunter, J. T., Okuda, T., Schlager, S. I., Rapp, H. J. and Borsos, T.: Local chemotherapy of a metastasizing guinea pig tumor. Proceedings of the Workshop on Cancer Invasion and Metastasis, 1976, Raven Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08552-11 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Complement Fixation and Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Langone	Senior Staff Fellow	LIB NCI
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	M. D. P. Boyle	Expert	LIB NCI
	H. J. Rapp	Chief	LIB NCI
	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a long-range project investigating the mechanism of complement fixation and action. In particular the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To apply rational complement fixation tests based on the fixation and transfer of the first component of complement and cytotoxic tests to the analysis of antigen-antibody reactions, in particular in the search for cancer specific antigens.

Methods Employed: The model for studying cytotoxic reactions mediated by antibody and complement consists of sheep erythrocytes, hemolytic antibody and guinea pig complement. Purification procedures for antibodies and the complement components include: preparative (large-scale) gel filtration, ion exchange chromatography and preparative free electrophoresis. Other techniques used include precipitin and immunoelectrophoretic analysis, analytical, zonal and preparative ultracentrifugation and other immuno- and physico-chemical methods.

Major Findings: The ultimate goal of this work is the development of immunological tools that permit exploration of 1) immunological approaches to cancer diagnosis and 2) possible host immune defense mechanisms against cancer.

Concanavalin A (Con A) inhibits killing in vitro of antibody sensitized line-1 guinea pig tumor cells by guinea pig complement (GPC), but not by human complement (HuC). Inhibition is dependent on the dose of Con A and is reversible. Con A appears to act by inhibiting binding of GPC1 but not HuC1 to sensitized line-1 cells. In contrast, Con A treated sheep erythrocytes (E) sensitized with the same anti-Forssman antibody (EA) fix the same amount of GPC1 as EA that have not been treated with Con A.

The effect of protein A and several lectins (e.g. Con A and wheat germ agglutinin) on the functional activity of the individual components of GPC or HuC and on hemolytic or tumor-specific antibody activity is under investigation. In the hemolytic reaction, Con A inhibits the activity of GPC1 and C2 but not C4 in the fluid phase; Con A has no significant effect once the components are bound to the cell. Con A inhibits the C-dependent lytic action of IgM but not IgG antibody, while protein A inhibits IgG but not IgM. These relationships hold with either tumor-specific antibody against line-1 or line-10 cells or anti-Forssman antibody used against the tumor cells or E.

In a new series of experiments, radiolabelled protein A is being used to develop a sensitive quantitative assay for IgG antibody on the surfaces of line-1 or line-10 cells and in the physiological fluids of animals inoculated with these tumor cells.

Significance to Biomedical Research and the Program of the Institute: Complement fixation is one of the most widely used diagnostic tools. The development and successful application of a very sensitive complement fixation test, the C1FT test, opened up new possibilities in determining antibody-antigen reactions on cell surfaces.

Furthermore, cytotoxic reactions due to antibody and complement are prime examples of body defense mechanisms. Fundamental research into the nature and mechanism of complement fixation and action will contribute greatly to the development of diagnostic tools and to the understanding of the mechanism of immune body defenses.

Proposed Course of Project: This is a long-range project, and little change is expected in the scope of the work during the next few years. The ultimate goals of this project are the development of better diagnostic tools based on fixation of complement and the elucidation of molecular events associated with the action of complement and antibodies. It is hoped that as a result of our program of inquiry into the basic problem of the interaction of antibodies, antigens and components of complement, tools will be developed that are of practical significance in the search for cancer antigens.

Publications

Boyle, M. D. P., Langone, J. J., Ohanian, S. H. and Borsos, T.: Effect of concanavalin A on the killing of tumor cells by antibody and complement. J. Immunol. 118: 1626-1630, 1977.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Detection of complement dependent antibody to tumor cells in sera of strain-2 guinea pigs cured of their tumors by BCG treatment. J. Natl. Cancer Inst. 56: 623-626, 1976.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Effect of concanavalin A on the classical complement pathway. J. Immunol. 118: 1622-1625, 1977.

Langone, J. J., Boyle, M. D. P. and Borsos, T.: Effect of concanavalin A on the functional activity of hemolytic antibody. Immunol. Commun. 6: 283-296, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08553-08 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immune Cytolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. D. P. Boyle	Expert	LIB NCI
OTHER:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
	S. I. Schlager	Staff Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the molecular aspects of the killing of nucleated cells by immune attack. In particular the interaction of antibody (class, affinity, etc.) and complement with one another and with the cell surface.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: A study of the molecular aspects of antibody-complement lysis and antigen topography of nucleated cells.

Methods Employed: Antibodies to normal and tumor antigens are detected and quantitated by immune cytolysis, colony inhibition, C1 fixation and transfer (C1FT) and by uptake of ^{125}I labelled antibody.

Immunochemical methods including Sephadex and DEAE chromatography, electrophoresis, immunodiffusion and ultracentrifugation are employed to isolate antibody protein and complement components.

A cytotoxic assay based on the release of ^{125}I from $^{125}IUdR$ prelabelled tumor cells has been developed. This test is superior to ^{51}Cr release cytotoxicity tests in that very little spontaneous release of isotope occurs over extended time periods and close to 90% specific release occurs from cells damaged by immune attack.

Major Findings: The malignant guinea pig cells, line-1 and line-10, appear to express less Forssman antigen than their normal tissue counterparts. Both line-1 and line-10 appear to have the same amount of Forssman antigen. Line-10 sensitized with anti-Forssman antibody is resistant to lysis by guinea pig complement (GPC) while line-1 is not. By C1FT tests, both line-1 and line-10 sensitized with anti-Forssman antibody have the same number of C1 fixing sites per cell.

The isolation of an intermediate in the killing of line-1 tumor cells by antibody and HuC or GPC has been achieved. This intermediate, called T*, is a tumor coated with antibody and all the necessary components of complement required for killing to occur. The formation of T* is dependent upon time and temperature of incubation of antibody coated cells with fluid phase complement. T* were only formed on addition of the entire sequence of complement components (1-9). Addition of the complement components 1 through 8 to antibody sensitized line-1 cells resulted in the release of ^{86}Rb. This was not a lethal event; the line-1 cells were capable of excluding trypan blue and their ability to synthesize DNA or reincorporate ^{86}Rb was not impaired.

Studies of ^{86}Rb release from line-10 cells on addition of antibody and GPC demonstrated significant levels of ^{86}Rb release without trypan blue uptake. Similar levels of ^{86}Rb from line-10 cells with antibody and HuC could be produced but in this case approximately 30% of the cells were stained with trypan blue. To further analyze the difference in efficiency between HuC and GPC the effect of adding HuC components to GPC was tested. Combinations of GPC with human sera deficient in C2, C5, C6, C7 or C8 suggested that GPC could provide the initial complement components for killing of antibody sensitized line-10 cells but that the later acting complement components in GPC were not as effective as those in HuC. Methods are being devised to measure the quantity of the later acting components of GPC or HuC bound to tumor cells. In order to develop such assays, rabbit antibody to GP or Hu 5, 8 and 9 have

been produced; these reagents would permit the analysis of the terminal stages of immune hemolysis or immune cytolysis.

Significance to Biomedical Research and the Program of the Institute. The elucidation at the molecular level of the effects of antibody and complement on both normal and malignant cells may be important in understanding control of cell growth by immunologic mechanisms.

Control of tumor cell growth in vitro makes it possible to study the action of specific and nonspecific agents on the cells at different stages of their growth cycle. The knowledge obtained from in vitro experiments will be very helpful when applied to the study of more complicated in vivo conditions.

Proposed Course of Project: The effect of antibody and complement on nucleated cells will be further analyzed. This will include studies of the class of antibody (both Forssman and antitumor) and binding of complement components, and of the physical and biochemical events that occur during formation and transformation of T*.

Publications

Boyle, M. D. P. and Ohanian, S. H.: Quantitation of antibody-complement mediated lysis of tumor cells. J. Immunol. Methods 15: 135-146, 1977.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Lysis of tumor cells by antibody and complement. VII. Complement-dependent ^{86}Rb release--A nonlethal event? J. Immunol. 117: 1346-1350, 1976.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Studies on the terminal stages of antibody-complement-mediated killing of a tumor cell. I. Evidence for the existence of an intermediate, T*. J. Immunol. 116: 1272-1275, 1976.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Studies on the terminal stages of antibody-complement-mediated killing of a tumor cell. II. Inhibition of transformation of T* to dead cells by 3'5' cAMP. J. Immunol. 116: 1276-1279, 1976.

Boyle, M. D. P., Ohanian, S. H. and Borsos, T.: Studies on the terminal stages of antibody-complement mediated killing of a tumor cell. III. Effect of membrane active agents. J. Immunol. 117: 106-109, 1977.

Ohanian, S. H. and Borsos, T.: Killing of nucleated cells by antibody and complement. In Day, N. K., and Good, R. A. (Eds.): Biological Amplification Systems in Immunity. New York, Plenum Publishing Corp., 1977, pp. 115-135.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08554-07 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Isolation and Study of Tumor-Specific Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Borsos	Head, Humoral Immunity Section	LIB NCI
OTHER:	Y. Keisari	Visiting Fellow	LIB NCI
	J. J. Langone	Senior Staff Fellow	LIB NCI

COOPERATING UNITS (if any)

None

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Laboratory of Immunobiology, Immunology Program

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Humoral Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Identification, quantitation, isolation and characterization of tumor cell specific antigens are of importance in several areas of cancer research. Knowledge about these would aid cancer diagnosis, studies of the carcinogenic process, cell transformation, and cancer immunotherapy, among others. The present study concerns the isolation, purification and immunochemistry of soluble tumor antigens of transplantable guinea pig hepatomas.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To obtain tumor-specific antigens in soluble and partially purified form. To use these antigens in inducing, establishing and measuring tumor-specific immunity in guinea pigs. Knowledge derived from the guinea pig model will be applied to the isolation, purification and characterization of human tumor-specific antigens. These antigens will then be used in diagnostic studies of human tumor epidemiology, in monitoring patient response to treatment and as an aid in treatment. Initial studies will be on the ascites cell form of diethylnitrosamine-induced hepatoma of strain-2 guinea pigs.

Methods Employed: Source of antigen: ascitic fluid of tumor-bearing animals or extracts of cell surfaces with 3M KCl. Further purification: ammonium sulfate precipitation, gel filtration, DEAE chromatography. Assay methods: skin testing; in vitro correlates of cellular immune responses, immunodiffusion, immunization of guinea pigs with extracts and challenge of test animals with live tumor cells. Production and testing of heterologous antisera to tumor antigen: immunization of rabbits with whole tumor cells, absorption of sera with various cell suspensions, testing of absorbed sera by C1 fixation and transfer test, by immune fluorescence, and by cytotoxicity.

Major Findings: Purification of soluble line-10 tumor antigens from ascitic fluid of ascites tumor-bearing guinea pigs continued. A major tumor specific antigen component (as detected by inhibition of cytolytic antibody) was found in the excluded portion of Sepharose 4B columns; the first included fraction contained a second tumor antigen not detectable by inhibition of cytolysis. In order to obtain antisera with high antibody content to these tumor antigens, rabbits were immunized with homogenized precipitate lines developed between line-10 antibody and line-10 antigens in double diffusion experiments in agarose. These antisera are now under intensive study and ultimately will be used to develop immunoabsorbent columns; these in turn will permit further purification of the antigen(s).

Significance to Biomedical Research and the Program of the Institute: The studies on the "naturally solubilized" antigens permit further characterization and immunochemical analysis of the line-10 and line-1 tumor antigens. Their presence in the tumor-bearing host and its immune response to them could offer one approach for a better understanding of the immune events in host-tumor interactions.

Proposed Course of Project: Further studies on the molecular nature of the soluble antigen and study of the biological significance of the presence of soluble antigens in the tumor-bearing host.

Publications

Detrick-Hooks, B., Smith, H. G., Bast, R. C., Jr., Dunkel, V. C. and Borsos, T.: Naturally soluble tumor antigens from guinea pig hepatomas: Isolation and partial characterization. J. Immunol. 116: 1324-1331, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 08555-05 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Detection of Complement Components on Nucleated Cell Surfaces

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. D. P. Boyle	Expert	LIB NCI
OTHER:	S. H. Ohanian	Head, Electron Microscope Unit	LIB NCI
	T. Borsos	Head, Humoral Immunity Section	LIB NCI
	J. J. Langone	Senior Staff Fellow	LIB NCI

COOPERATING UNITS (if any)

Institut fur Immunologie der Universitat Heidelberg, Heidelberg, Germany

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop sensitive immunochemical methods for the quantitation of complement components on a molecular basis. The techniques will be applied to determine the amount and persistence of selected complement components bound to the cell membrane or tumor cells grown in vivo.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To determine whether tumor cells of human (Hu) and guinea pig (GP) origin have detectable complement (C) components on their cell surfaces; 2) to develop techniques for the detection of other complement components bound to the cell surfaces.

Methods Employed: Immunochemical methods including DEAE chromatography, electrophoresis and immunodiffusion are employed to isolate antibodies, protein and complement components. Quantitation of antibody and complement components are carried out using radioisotopic labelling, quantitative precipitin procedures, C1FT tests, and immunochemical tests designed to measure C4 and C3.

Major Findings: Hu and GP C4 and C3 can be bound to the surface of sheep erythrocytes and remain bound for up to two weeks. A test based on inhibition of lysis of these cells is capable of detecting as little as 6×10^{11} Hu and GP C4 molecules and 10^{11} Hu and GP C3 molecules. Using these methods, it has been possible to detect C4, C3 and host immunoglobulins bound to the surface of two diethylnitrosamine-induced guinea pig hepatoma cell lines designated line-1 and line-10. The quantity of C3, C4 and immunoglobulin detected was dependent on the time of collection of the tumor cells (after intraperitoneal injection). C4, C3 and immunoglobulin were lost from the tumor cell surface during incubation in tissue culture. The loss of the cell bound components occur at 4°C and 37°C with the greatest loss being at 37°C.

Currently methods are being developed to quantitate the later acting complement components bound to the tumor cell surface. Antibodies to GP and Hu C5, C8 and C9 have been prepared in rabbits and it is hoped that these reagents can be used to provide the basis for measuring the levels of cell bound and serum C5, C8 and C9. The terminal stages of immune hemolysis are being studied to obtain reliable methods for quantitation of the number of hemolytically effective C8 or C9 molecules present in a fluid phase complement source.

Significance to Biomedical Research and the Program of the Institute: Techniques for detecting bound complement components may be useful in determining whether a humoral immune response has been produced to tumor cells in vivo. The ability to detect minute quantities of antibodies to tumor cells may provide means of relating humoral immune responses to antitumor effects in vivo.

Proposed Course of Project: Techniques will be developed to detect complement components other than C3 and C4 bound to tumor cell surfaces. In particular emphasis will be placed on developing assays for the quantitation of cell bound C8 and C9. Additional studies concerning the fate of cell bound immunoglobulin will be carried out using the existing methods and also utilizing radiolabelled protein A as another probe for studying 7S immunoglobulin bound to the tumor cell membrane.

Publications

Borsos, T. and Rother, U.: Deviated lysis: III. Kinetics of interaction of DL with chicken erythrocytes: Evidence for E* formation. Z. Immunitaetsforsch. 153: 36-47, 1977.

Segerling, M., Ohanian, S. H. and Borsos, T.: Persistence of immunoglobulin and complement components C4 and C3 bound to guinea pig tumor cells. J. Natl. Cancer Inst. 57: 145-150, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08556-01 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Ultrastructural Studies of Nucleated Cells Following Modification by Physical or Chemical Means

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. H. Ohanian Head, Electron Microscope Unit LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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Humoral Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine what ultrastructural changes occur in tumor cells following modification with chemotherapeutic drugs, enzymes, hormones or following humoral immune attack. The techniques of Scanning Electron Microscopy and Transmission Microscopy will be employed.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To determine the ultrastructural modifications occurring in tumor cells undergoing humoral immune attack, cellular immune attack and chemical or physical treatment which increases their sensitivity to immune attack; 2) to develop techniques for the localization of antibody and C binding sites on tumor cell surfaces.

Methods Employed: Antibodies to antigens of tumor cells and selected C components will be purified and labelled for detection in electron microscopy. Histochemical, immunohistochemical and immunoelectron microscopy techniques will be employed.

Major Findings: Procedures for the fixation of tumor cells in suspension or growing in the tissue of guinea pigs have been developed.

Tumor cells killed by humoral immune mechanisms appear enlarged and devoid of villi. Preliminary results of experiments with samples evaluated at different times after addition of C to antibody sensitized cells show a sequence of events in membrane pertubations. Local swelling or blebbing occurs at localized areas of the membrane followed by swelling of the cell and loss of villi. The surface of the cells killed by antibody plus C is extensively damaged compared to dead cells in control preparations.

Preliminary results of SEM examination of tumor cells growing in the skin and lymph node of guinea pigs indicate the morphology of the cell is similar to that of cells grown in the peritoneal cavity. Chemotherapeutic drugs injected into intradermal growing tumor cells induce minimal changes in surface morphology of the cells.

Significance to Biomedical Research and the Program of the Institute: These studies are designed for analyzing the ultrastructural changes which occur in tumor cells following treatments which are effective in rendering the cells sensitive to immune attack. Visualization of physical attributes of tumor cells which may play a role in the resistance of tumor cells to immune attack should help in the understanding of defense mechanisms to immune attack.

Proposed Course of Project: Analytical studies of morphological changes occurring in tumor cells following antibody-C mediated attack will be expanded. Purified antibodies specific for Forssman and tumor antigens will be labelled with marker molecules for localization of these antigens on tumor cells. Characterization at the SEM and TEM level, of pathological features of tumor growth in vivo and of modifications following treatments with various chemical or physical agents.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 08575 05 LIB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. J. Leonard	Head, Immunopathology Section	LIB	NCI
	M. S. Meltzer	Surgeon	LIB	NCI
OTHER:	S. Watson	Visiting Fellow	LIB	NCI

COOPERATING UNITS (if any)

None

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Immunopathology Section

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NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to study the cells that participate in the effector arm of the immune response. The current emphasis is on chemotaxis, which is a mechanism by which cells can be attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes histology of different types of delayed hypersensitivity lesion, chemistry of lymphocyte derived chemotactic factors and identification of substances that modulate chemotactic responses.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To develop quantitative measures of reactions occuring in the effector limb of the immune response; to study chemotaxis of inflammatory cells in relation to this objective.

Methods Employed: Complement-derived and lymphocyte-derived chemotactic factors were generated. Human peripheral blood leukocytes were separated on Ficoll-Hypaque gradients to obtain monocyte- and basophil-rich fractions. Peritoneal macrophages were harvested from normal and BCG-infected mice. Protein fractionation methods were employed to characterize chemotactic factors and serum factors that modulate mouse macrophage chemotactic responses. Delayed hypersensitivity reactions were induced in guinea pigs. Histological study of the reactions was made in sections processed to preserve basophil morphology.

Major Findings: 1. Modulation of macrophage motility. It was shown last year that chemotaxis of mouse peritoneal macrophages required serum in the cell suspensions and that the serum effect was mediated by a single macromolecule with a M.W. of about 100,000. MSP (macrophage stimulating protein) had been further purified in a series of steps that includes G-200 Sephadex and DEAE chromatography, isoelectric focusing (P.I. = 7.0), absorption with Cowan strain S. aureus to remove traces of IgG, and polyacrylamide gel electrophoresis. MSP activity per mg protein was increased about 400-fold. The protein MSP activity in whole serum is mediated-by less than 0.5 ug/ml of the protein.

2. Basophils in inflammation. The composition of the leukocyte infiltrate in delayed cutaneous hypersensitivity lesions of guinea pigs is determined by the mode of immunization. In the classical tuberculin reaction, basophils are rare; whereas the response to hemocyanin in animals primed with hemocyanin in saline is a lesion rich in basophils. There are more basophils in one such lesion than in the total circulation blood volume of the guinea pig. Therefore antigenic challenge must initiate a remarkable sequence of events that signal the release and perhaps maturation of basophils from bone marrow or as yet unknown sites.

Human peripheral blood basophils migrate toward chemotactic factors, including complement C5a and substances in culture fluids of stimulated lymphocytes (LDCF). Addition of culture fluids to basophil suspensions increased the chemotaxis of basophils to C5a. The activity in the culture fluids was called basophil chemotaxis augmentation factor (BCAF). It is a macromolecule with a molecular weight of about 30,000. It was separated from LDCF, which has a M.W. of about 12,000. Experiments with the purified fractions showed that BCAF increased basophil chemotaxis to LDCF as well as C5a.

Among the interesting aspects of basophil cell biology are (1) only a fraction of the blood basophils migrate toward chemotactic factors, but the size of the fraction can be increased rapidly by BCAF. Other evidence for basophil subpopulations is the inhibition of migration by histamine,

affecting all but a fraction that is not inhibited by even high concentrations of the drug. (2) Addition of C5a to basophils, followed by washing, inhibited chemotaxis to C5a but did not affect chemotaxis to LDCF. Similar results were obtained by addition of LDCF to basophils. This shows the specificity of receptors for chemotactic factors. (3) Histamine inhibits histamine release from basophils. The chemotactic response of basophils to C5a was inhibited by low concentrations of histamine. It would be of interest to know whether this constitutes a negative feedback control of basophil accumulation at sites of inflammation. The effect of histamine was abolished by prior addition of H2-receptor inhibitors.

Significance to Biomedical Research and the Program of the Institute: The monocyte or basophil chemotactic response is a useful measure of one function of the effector arm of the immune response, which has been shown to be depressed in cancer patients. The findings that there is not a basophil-specific chemotactic factor shows that other explanations must be sought for the perferential accumulation of basophils in certain types of hypersensitivity skin reactions. The previously described basophil chemotaxis augmentation factor, which is produced by stimulated lymphocytes and enhances basophil responses to complement-derived chemotactic factor, may account for preferential accumulation. The characterization of mouse lymphocyte derived chemotactic factor shows striking chemical differences from the functionally comparable activity produced by lymphocytes of other species. It also allows for comparison with chemotactic factor produced by mouse neoplasms, described in another project report from this Section. The finding of a serum protein needed for mouse macrophage responsiveness to chemotactic factor shows that there may be regulatory serum factors that modulate macrophage motility.

Proposed Course of Project: Further study of mechanisms of basophil chemotactic response, including chemical characterization of basophil chemotaxis augmentation factor. The effect of the chemotaxis promoting factor of human serum on other aspects of macrophage function.

Publications

Leonard, E. J. and Meltzer, M. S.: Characterization of mouse lymphocyte-derived chemotactic factor. Cell. Immunol. 26: 200-210, 1976.

Leonard, E. J. and Skeel, A. S.: A serum protein that stimulated macrophage movement, chemotaxis and spreading. Exp. Cell. Res. 102: 434-438, 1976.

Lett-Brown, M. A., Boetcher, D. A. and Leonard, E. J.: Chemotactic responses to normal human basophils to C5a and to lymphocyte-derived chemotactic factor. J. Immunol. 117: 246-252, 1976.

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Meltzer, M. S., Stevenson, M. M. and Leonard, E. J.: Characterization of macrophage chemotaxis in tumor cell cultures and comparison with lymphocyte-derived chemotactic factors. Cancer Res. 37: 721-725, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08576 05 LIB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunological Mechanisms of Tumor Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. J. Leonard	Head, Immunopathology Section	LIB	NCI
	M. S. Meltzer	Surgeon	LIB	NCI
OTHER:	L. Ruco	Visiting Fellow	LIB	NCI
	R. Aksamit	Staff Fellow	LIB	NCI

COOPERATING UNITS (if any)

None

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SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is on the interaction of tumor cells and host defense cells. Current studies include the depression of macrophage chemotaxis that occurs in tumor-bearing patients and experimental animals; the increase in chemotactic and tumoricidal activity that occurs in BCG-infected tumor-bearing mice; the characterization of a chemotactic factor produced by growing tumors in vitro, which could lead to accumulation of macrophages in tumor sites; and the isolation of lymphotoxin, a protein produced by mitogen or antigen-stimulated lymphocytes that is toxic for tumor cells.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To determine what immunological mechanisms occur in tumor cell killing in order to provide a rational basis for elimination of tumor cells by immunological means.

Methods Employed: Chemically-induced murine tumors, maintained in tissue culture or by passage in syngeneic hosts were used. Effector immune responses were measured by (1) cytotoxicity assays based on the release of radioactive label from damaged target cells and (2) macrophage chemotactic response to complement or lymphocyte-derived chemotactic stimuli.

Major Findings: 1. Induction of cytotoxic macrophages in vivo. Macrophages play a major role in host defense against neoplastic disease. These cells, or a functionally activated subpopulation, have antitumor activity. Mice injected intraperitoneally with BCG develop a chronic infection in which tumoricidal macrophages can be recovered by 1 week and persist for more than 40 weeks. The tumoricidal cells are localized at the site of BCG challenge (peritoneal cavity). Mice infected intradermally do not develop cytotoxic peritoneal cells, although the mice become immune to BCG antigens. Intraperitoneal injection of soluble BCG antigens (PPD) into these mice induced tumoricidal macrophages. Cytotoxic cells were found by 9 hours after antigen injection and persisted 2 days. Studies with a cytochemical marker for peroxidase showed an increase from a very low to a very high percentage of peroxidase-positive macrophages after PPD challenge. The peroxidase-positive cells are cells that have recently migrated into the peritoneal cavity. These newly immigrated cells were not sufficient for tumor cytotoxicity, since peroxidase-positive cells induced by irritants were not cytotoxic. Induction of cytotoxic macrophages by antigen required both a macrophage precursor cell and a specific immune response.

2. Induction of cytotoxic macrophages in vitro. Peritoneal macrophages from normal mice were activated to become cytotoxic after adoptive transfer in vivo of immune spleen cells and specific antigen. Similarly, incubation in vitro of peritoneal macrophages from normal mice with immune spleen cells and specific antigen induced tumoricidal macrophages. In vitro activation of normal macrophages also occurred with supernatants from antigen-stimulated immune spleen cell cultures (lymphokines). Tumoricidal activity was evident after 3 hours of culture in lymphokines, was optimal at 812 hours, and disappeared at 20 hours. Also, the capacity of normal macrophages to be activated by lymphokines progressively decreased with time in culture and was absent at 20 hours. The loss of capacity for activation was not due to cell death. After PPD injection intraperitoneally to BCG immune mice, cytotoxic cells appeared and then disappeared within 20 hours of antigen clearance. The loss of cytotoxic cells reflects the short-lived nature of both the cytotoxic capacity of activated macrophages and the capacity of precursor cells to be activated by lymphokines.

3. Correlates of macrophage activation for cytotoxicity. Several functional properties distinguish activated tumoricidal macrophages from normal macrophages or macrophages elicited by irritants. These include increased

response to chemotactic stimuli and release of a soluble monokine that was mitogenic for T-lymphocytes (LAF). BCG-activated macrophages released 3-5 times more LAF than equal numbers of normal macrophages. Macrophages from mice treated with thioglycollate or mineral oil were not cytotoxic and did not produce increased LAF.

4. Lymphotoxin. The term lymphotoxin has been applied to the factors in supernatant fluids of stimulated lymphocyte cultures that destroy tumor cell lines, although cell counting assays for lymphotoxin may detect factors that inhibit proliferation as well as those that kill. An assay of cell killing is release of thymidine from prelabelled target cells; it has been used in this laboratory for isolation and purification of mouse lymphotoxin. Further study of the conditions for LT assay have shown (1) the sensitivity of cells to LT action was greatly increased by tritiated thymidine labelling. This was due to the radiation, not to thymidine. (2) Addition of DNA-ase, as late as 15 min before the end of the 72 hour assay, doubled tritium release. After the death of cells, release of nuclear thymidine is not inevitable and can be increased by manipulation of culture conditions. (3) LT was equilibrated with target cells for 24 hours and then removed; there was no thymidine release at 24 hours. The chain of events leading to cell death and thymidine release continued despite removal of LT. Counts released at 72 hours were at least 60% of the counts released after 72 hours with LT. (4) There was no detectable absorption of LT by target cells, as determined by titration of LT remaining in fluids after equilibration with target cells. This suggests weak binding to target cell receptors.

Significance to Biomedical Research and the Program of the Institute: Analysis of the effector immune events leading to tumor rejection may provide a rational basis for manipulation of host responses or tumor cell in an effort to eliminate or prevent progression of the tumor.

Proposed Course of Project: Studies on the mechanisms of the killing of tumor cells by macrophages; on the factors modulation macrophage chemotaxis, especially in tumor-bearing mice; further characterization of mouse lymphotoxin and mouse chemotactic factors of lymphocyte and tumor origin.

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Annual Report of the Laboratory of Cell Biology

National Cancer Institute

July 1, 1976 - September 30, 1977

Viral Oncology Studies

A major thrust of this study is to elucidate the basis of neoplastic transformation of normal tissues. Two experimental model systems were used in this project: (1) a rat hepatoma induced by the chemical carcinogen N,N-2, 7-fluorenylene bis-2,2,2-trifluoroacetamide, which has been adapted to tissue culture (HTC cells); and (2) two intracisternal A particle (IAP) containing neoplasms, a plasma cell tumor (MOPC-104E) and differentiated neuroblastoma, also adapted to tissue culture (cell lines N-4, N-18, and NB-2A). Multidisciplinary approaches were required in this investigation. They involve tissue culture, biochemical and biophysical analyses, cytogenetics, immunology and tumor biology. Areas with special emphasis of research effort are: (1) extensive characterizations morphologically biologically, biochemically and immunologically of KiMSV(RHHV), a transforming viral complex successfully rescued out of a co-culture of rat hepatoma tissue culture cells (HTC-H1) with K-NRK cells; (2) regulation of viral gene (GAG for gs antigens and ENV for envelope antigen) expressions; (3) the oncogenic potential of KiMSV(RHHV); (4) virus infection and the development of chromosome abnormalities during the process of neoplastic transformation, and (5) integrations of viral genome and its localization on host cell chromosome(s).

Although much work has been done in the areas of tumor biology the basis of how tissues come to lose their normal cellular control mechanism over metabolism and develop into the neoplastic state remains to be elucidated. Our major effort has been concentrated toward a better understanding of neoplastic transformation by adopting as an experimental model system the chemically induced rat hepatoma, in tissue culture, called HTC cells. During the past year extensive efforts in genome rescue experiments resulted in the isolation of a rat heptoma virus pseudotype Kirsten sarcoma virus, KiMSV(RHHV), when HTC-H1 cells were co-cultivated with Kirsten sarcoma virus transformed non-producer rat kidney cells (K-NRK) (J. Gen. Virology 1976, 31, 239-243). As an on-going project characterizations biologically, morphologically, biochemically and immunologically of KiMSV(RHHV) have now been completed. It has a C-type virus ultrastructure. It is strictly rat tropic in host range. The helper virus, RHHV, originates from the hepatoma cells as suggested by the mode of its rescue and by its host specificity. KiMSV(RHHV) sediments isopycnically in a linear sucrose gradient at 1.145-1.155 g/cm^3, a lesser density than that of the mouse C-type tumor viruses (1.160 g/cm^3). It also possesses the complements of RNA and reverse transcriptase. Results obtained by indirect fluorescent-antibody assays and neutralization bioassays indicate that KiMSV(RHHV) exhibits a major rat leukemia virus (RaLV) specific antigen (GP 70, glycoprotein of 70,000 mol. wt.) and an inter-mammalian group specific gs-3 (P30, 30,000 mol. wt. protein) antigen.

The major viral proteins associated with KiMSV(RHHV), labelled in either ^{35}S-methionine or ^{3}H-amino acids, were analyzed by 5-30% gradient polyacrylamide gel electrophoresis. They consist of the GAG gene products peaking at the 70,000 mol. wt., the 30,000 mol. wt. (P30), and an 8-10,000 mol. wt. (P10) area. A major peak of radioactivity was observed at the 200,000 mol. wt. area; it is a glycosylated protein as discussed below. Incorporation studies with ^{3}H-glucosamine and ^{14}C-galactosamine showed that these precursors for glycoprotein were incorporated into a large molecule fraction of 200,000 mol. wt. and also a protein fraction of 70,000 mol. wt. Origin of the high mol. wt. glycoprotein 200,000, whether from cell or from virus, has yet to be determined. The latter fraction is the ENV gene product, gp 69-70. Currently we are concentrating on the regulation of synthesis and assembly of these major viral gene products by radioactive pulse-chase experiments using virus infected cells. The results of these experiments are compared to those obtained in a cell-free protein synthesis system with transcribed mRNA dictated by viral cDNA transcribed from the 5'OH end of the viral genome using KiMSV-(RHHV) reverse transcriptase. By these studies we hope to resolve the order of GAG and ENV genes in the viral genome and also the control of their expressions.

In a long term project we have been investigating the possible oncogenic and immunogenic effects of KiMSV(RHHV) in vivo. Inoculation of 10^{2} to 10^{3} FFU of KiMSV(RHHV) into newborn Fisher rats did not produce any pathological change among some fifty animals examined at different periods from 14 day p.i. to 15 months p.i. We are currently also examining the presence of specific antibodies in the sera of the inoculated rats after additional inoculations of KiMSV(RHHV). Further studies have also been pursued with thymetomized newborn rats along the same scheme.

In contrast, we have observed that when KiMSV(RHHV) transformed Fisher rat embryonic cells were inoculated into syngeneic newborn or young adult rats, not only was there rapid growth of a capsulated tumor at site of inoculation, but metastases were also seen primarily to the liver. The pathology of such capsulated tumors, as determined from the histological studies of serial sections of the tumor specimens, was minimally differentiated carcinoma.

An ecotropic virus designated Dl-MuLV has been isolated and characterized as reported previously. Since the etiologic role played by this virus in RCN is not yet clear, another endogenous virus which is xenotropic in its tropism was looked for in the SJL/J strain of mouse. After many trials, we finally succeeded in isolating a xenotropic virus from the normal mouse embryo cultures and RCN cultures of SJL/J mouse by co-cultivation with Kirsten MSV-transformed nonproducer human osteosarcoma cell (KHOS) and subsequent propagation in a line of rabbit cornea cells (SIRC). The virus was compared with other known xenotropic viruses such as those from NZB mouse embryos, and AT124 virus that was isolated from NIH Swiss mice. They were found to be closely related by cross interference tests and neutralization tests, although minor differences may exist. Further work is in progress to characterize this virus and investigate its role in oncogenesis and immunologic reactivities of RCN.

The D1-MuLV RNA has been extracted, and a complementary DNA (cDNA) was prepared. This will serve as a probe for determination of the degree of homology between the RNA of other murine retroviruses such as Rauscher, Moloney, and Gross, or those from other animal species. This probe will also be used to investigate the presence of D1-MuLV genome in various tumor as well as normal cells. This work is in progress.

Intracisternal A particles. In a continuing study of mouse intracisternal A particles (IAP), it has been established in a collaborative study with Dr. Raymond Gantt, that such particles apparently have a number of tRNA methylases. It has been previously shown that IAP have a number of biochemical markers which are characteristic of RNA tumor viruses. Appropriate experiments indicate that the tRNA methylase specific activity is highest in purified preparations of IAP and that it is bound within the particles rather than simply being associated with the outer surface. The latter finding is documented by the fact that detergent treatment of IAP with 1% Triton does not remove tRNA methylase activity, although this concentration of detergent is sufficient to remove the outer shell of IAP. Current efforts are directed toward a comparison of the IAP-associated tRNA methylases with those of the host cell. The results should give a strong indication about whether these methylases are coded for by the particle genome or whether they are simply cell methylases which are incorporated during the process of replication in a manner analogous to incorporation of cell actin by some budding enveloped viruses.

The endogenous DNA product of IAP reverse transcriptase was analyzed in detail by various physicochemical methods. Nascent DNA transcript of IAP reverse transcriptase appeared to be covalently linked to an RNA primer. Fidelity of transcription is evident since the DNA transcript hybridized specifically back to the 35-70S RNA of IAP but not with heterologous RNAs. The DNA transcript has an approximated M.W. of 145,000, estimating 350 polynucleotides and base ratios with an excess of dGMP.

Interferon treatment of murine oncornaviruses. Continuing studies with Dr. Paula Pitha have added to our knowledge about the effects of interferon on in vitro AKR virus infection. There appears to be a differential effect exhibited by interferon which is dependent on the host cell used to replicate the virus. When AKR-L1 cells are infected with AKR virus and then treated with interferon, there is a block in virus assembly with arrest at the budding stage. Thus there is a significant inhibition of virus release from the cell, and the virus titers in the tissue culture medium are reduced accordingly. When SC-1 cells, derived from a feral mouse and lacking FV-1 restriction, are infected with AKR virus and treated with interferon, the block in virus assembly does not occur at the budding stage, but whole virions remain trapped at the plasma membrane of the cell. Again the virus titer in the tissue culture medium is reduced. Trypsinization of the infected, interferon-treated SC-1 cells does not appear to release significant amounts of the cell-associated virus. However, the results seen with both AKR and SC-1 cells tend to indicate that the mechanism of interferon action is associated with membrane phenomena that are critical to a late stage of virus assembly and release. Since interferon could inhibit the translation

of a virus protein necessary for assembly, current efforts are directed toward the characterization of interferon-treated virions to determine if there are intrinsic polypeptides missing.

In the instance of the interferon-treated C-type viruses, there is an accumulation of either whole virus or viral buds at the cell surface. The data from the SEM-computer system confirm the situation in quantitative statistically significant terms. In a representative experiment, the infected control cells showed 2.67 particles per field of cell surface, while in the interferon-treated infected cells there were 10.67 particles per field of cell surface. It seems apparent that this automated approach is both feasible and accurate, and it has been confirmed by manually counting the fields instead of using the computer.

Transfer RNA from Mouse and Rat Tumors

The variations in isoaccepting tRNAs among tumors and normal tissues are being studied using: 1) reversed phase, high pressure liquid chromatography, 2) polynucleotide and triplet directed ribosome binding, 3) cloning in tissue culture, 4) utilization in cell free protein synthesis, and 5) RNA sequencing techniques.

We have determined that an extra, tumor-specific "Phe-$tRNA_1$" isolated from rat hepatoma 7777 differs from normal "Phe-$tRNA_2$" by not having the modified base "Y" which is characteristic of most eukaryotic Phe-tRNA. In order to determine how wide spread this "Y absence" is and how general a property this is for tumors we surveyed 22 rat neoplasms of different cell types, 20 mouse tumors of various origins, 11 mouse plasmacytomas, and 4 normal organs of rats and mice for the presence or absence of this early-eluting, Y-minus Phe-tRNA. Normal tissues never contained this Phe-tRNA even when extracts of embryonal or fetal liver were examined. Such a peak could be artificially generated from normal Phe-tRNA if buffering during tRNA preparation was inadequate. This artificial Y-minus Phe-tRNA is easily confused with the tumor-specific peak, but we developed a test using 3-methyl-2-benzothiazolinone hydrazone (MBTH) which reacts only with tRNA from which an entire base, such as Y, has been excised. 15/22 rat tumors had peaks of tumor specific Phe-tRNA (not reacting with MBTH); 12/21 mouse tumors surveyed had an MBTH unreactive tumor peak of Phe-$tRNA_1$, and in addition, none of the 11 lines of BALB/c plasmacytomas contained such a molecule. There was a significant difference between the tumor peaks of Phe-$tRNA_1$ in mice and rats. Those mouse tumors that were positive for this marker had a nearly complete replacement of their Phe-tRNA with this Y-minus tRNA, whereas the proportion of Y-minus Phe-tRNA in rat tumors never exceeded 20% of the total Phe-tRNA.

Tumor Antigens, Histocompatibility Antigens and Immune Responses

We have studied the distribution of TSTA in the membrane and nuclear fractions of mKSA sarcoma cells. The preparations obtained had to be free of cross-contamination (that is of TSTA and of T ag); as a consequence membrane marker and nuclear markers were employed. It was found that nuclei were far more effective in protecting BALB/c syngeneic mice against challenge with mKSA than the crude membrane preparation - the membrane containing 5-10 times

less TSTA than nuclei. In fact all of the activity of the membrane may be accounted for by contamination with the nuclear T antigen. The majority of TSTA of mKSA cells was consistently found in those fractions that also contained T ag (measured by a quantitative CF assay). Since T ag is not found on the cell membrane, it has been argued that TSTA and T ag are processed differently in the cytoplasm before traveling to the membrane and to the nucleus. We favor the concept, however, that T antigen on the membrane is the effective TSTA and is extremely unstable, with rapid turnover on the membrane. Obviously it is necessary to purify both T ag and TSTA to determine the relationship and the role of the viral DNA in the coding of these antigens.

Another method has been used to solubilize TSTA from the SV40 mKSA membranes - the use of the detergent Nonidet P-40 (NP-40). In contrast to the findings with papain a good recovery (39%) was achieved with solubilization. The soluble antigen was subjected to polyacrylamide-agarose gel filtration (AcA 22 ultrogel column) and tumor rejection activity of the various fractions as well as H-2 and β-2 microglobulin activities were followed. One fraction (V) contained 73% of the activity of the solubilized material whereas only 9% of H-2 activity was recovered in this fraction. The molecular weight range of this fraction was 40,000 - 80,000 daltons. The specific activity of the V fraction remained approximately the same as the crude solubilized material. A reasonable dose-response reactivity was found for the immunogenic fraction V as well as for the solubilized material and these fractions did not affect the growth of the syngeneic Meth-A sarcoma. Of interest is the finding that H-2 activity could be completely separated from TSTA activity through the use of an LcH affinity column.

Meth-A, a chemically induced sarcoma of BALB/c mice, contains strong individually distinct TSTA. This antigen has been solubilized from dissociated membranes using NP-40. This TSTA with specific tumor rejection activity has a MW of ~70,000 daltons (in the presence of detergent with an electrophoretic mobility of α-globulin. TSTA was found to separate from H-2. The soluble antigen was extensively purified by a sequence of procedures: gel filtration, lectin affinity chromatography, column electrophoresis. The specific activity of TSTA increased during the purification procedure and in the final step used, PAGE, only 3 major bands were detected. A very small amount of purified antigen 4 μg provided greater than 50% specific tumor inhibition in the syngeneic system and a good dose-response reactivity was attained.

An additional antigenic specificity was identified on Meth-A membranes through the preparation of a xenogeneic antiserum (in rabbits) using the partially purified TSTA as antigen and extensively absorbing the antiserum *in vivo*. This antigen designated, TSSA, was further purified with retention of both tumor rejection and TSSA activity (assayed by a radioimmunoassay). The TSSA antigen was shown to have a MW of 60,000 daltons and on electrophoretic mobility of α-globulin. Studies are in progress to determine the relationship between TSTA and TSSA.

Study has continued of TSTA of lymphocytic neoplasms induced by FMR viruses (but specifically RBL-5). The role of the viral structural entities, gp70 and p30, functioning as TSTA has been studied. These proteins, as well as p15, have been identified on cell surfaces by cytotoxic assays employing xenogeneic antisera and their role in prevention of leukemia (erythroblastosis?) and tumor rejection (in the rat principally) has been emphasized by several investigators.

TSTA solubilized from RBL-5 membranes were extensively digested with papain; this solubilized material retained specific tumor rejection activity and inhibitory activity in in vitro cytotoxic assays. More than 90% of the activity of p30 and gp70 was destroyed. Also, purified p30 and gp70 (obtained from Dr. S. Oroszlan) had no appreciable influence when used as immunogens in tumor rejection or as inhibitors of specific cell mediated cytotoxicity (in collaboration with Dr. C. C. Ting) we have not yet been able to study other translational products of the C-type genome such as p10, 15 and 12.

In extensions of this work a new technique of using a Stansted cell disruptor to obtain purified cell membranes has been introduced. Solubilization was achieved by Na deoxycholate (DOC) followed by chromatography on an AcA-34 agarose-acrylamide gel column. Nine separate fractions were assayed for tumor rejection activity against RBL-5 (and MCA-10, Meth-A and mKSA as specificity controls). The findings of significance were:

1). Fractions 1 (aggregated void volume) 2, 3 and 4 contained the major portion of tumor rejection activity. As low a concentration as 1 µg for example of fractions 2 and 3 provided greater than 50% tumor inhibition. A reasonable dose-response activity was obtained in the dosage range 0.5 µg to 50 µg.

2). Fraction 5, containing both H-2 and gp70 activity and the combined fractions 6 and 7 (containing major gp70 activity) provided only minimal tumor rejection activity.

3). Only fraction 8 material inhibited the cytotoxic complement-dependent activity of our syngeneic anti-crude membrane serum. This antiserum has been found capable of lysing purified Rauscher virus (S. Oroszlan). Since only antisera directed against gp70 and p15E are capable of lysing virus, fraction 8, therefore contains some p15E antigen. p30 has not been detected in any of the fractions. The bulk of the evidence obtained to date therefore favors the concept that there exists on MuLV-induced leukemic cells specific TSTA entities free of viral structural proteins.

Intensive work has been devoted to the purification and chemical characterization of H-2 antigens. Membranes have been prepared from liver cells by differential centrifugation according to established procedures. The membranes are digested by limited papain which solubilizes H-2. The solubilized H-2 is purified by DEAE, gel filtration, affinity chromatography on concanavalin, and isoelectric focusing. By inhibition of binding of radiolabeled antigen, this material has been shown to be about 80% pure. A single peak of about 40,000 could be obtained after full reduction and alkylation and filtration on G-100 Sephadex column. This might represent the purified heavy

chain of the H-2 molecules. A chemical analysis has shown a blocked amino-terminal. Peptide analysis is now in progress and a comparison with the chemical structure of HLA molecules should reveal new insights into the evolutionary relationship of these molecules and the immunoglobulins.

The role of H-2 antigens either in a modified form or existing as derepressed antigens in tumor rejection has been reported in the literature.

1). Meth-A soluble antigens are separated by chromatography and attempts to detect alien H-2 specificities on Meth-A cells has not been successful. It appears that, for this sarcoma, TSTA are distinct from H-2.

2). In collaboration with Dr. Parmiani (NCI, Milan) we have completed some preliminary investigation using a BALB/c chemically induced sarcoma having alien H-$2K^k$ specificities that appear to be the tumor rejection antigen (TSTA). It has been confirmed using papain solubilized antigens from this neoplasm C-1 (1) that a) the alien specificities do exist and are not artifacts (as determined by inhibition of cytotoxicity and a radioimmunoassay) and b) that the normal H-2D specificities do exist on the membranes and both are located in the same molecules purified from the membranes. This investigation will proceed but preparations of the membranes from C-1 (1) for adequate yield need to be done in this laboratory.

3). We have initiated a study using pedigreed BALB/c mice to induce a series (6) of sarcomas by methylcholanthrene. Individual TSTA have been identified (no cross reactivity) in all neoplasms. Each neoplasm is being studied for the existence of alien specificities. If detected by in vivo assays (through the use of F_1 hybrid recipients) membranes will be solubilized and specific H-2 specificities determined. The precise role of these can then be determined through fractionation procedures of the solubilized products.

A nonproducer cell line cultured from a hemangioma induced by Moloney-MSV in (C57BL/Ka X C3H)F_1 mice has been further investigated. This cell line (HIIHP) was shown to produce no type-C virus and no MSV rescuable by co-cultivation with mouse cells producing helper viruses. However, as published previously, this cell line possesses a tumor specific antigen which can be demonstrated by tumor rejection experiments in which HII tumor growth can be inhibited in M-MSV-immunized syngeneic recipients. Further experiments showed that this transplantation immunity was specific, in that the HIIHP-immunized syngeneic host could not reject a polyoma virus induced tumor, and the polyoma virus-immunized host did not reject HII tumor.

Further experiments were carried out to examine the virological and immunological characteristics of HIIHP cells. If M-MSV or M-MuLV genome is present in HIIHP, it may adversely influence the sensitivity of HIIHP cells to superinfection by M-MSV (M-MuLV) or closely related M-MSV (R-MuLV). This was found to be true in induction of transformation foci after superinfection with these pseudotype viruses. No resistance was observed against superinfection by Harvey-MSV or vesicular stomatitis virus. Furthermore, immunization of (BL/Ka X C3H)F_1 mice with various murine tumor cells such as HFL/d-tc (Friend virus-induced nonproducer tumor), RBL-3, and KiMSV-BALB/3T3 and NIH/3T3 FL gave a significant protection (measured by tumor incidence and/or

tumor size) against the challenge inoculation of HII tumor cells. Borderline protection was obtained with FBL-3 and no protection was observed with BALB/3T3, and M-MSV-NIH/3T3FL. These results may be correlated with the amount of expression of some VSP such as sp69/71 or TSTA of FMR specificity. Sequence homology studies by using cDNA of Moloney-RNA as a probe to detect the presence of Moloney-MuLV proviral DNA in HIIHP cell are in progress.

Immune Reactions of the Tumor Bearing Host

a). The host bearing a large > 25mm, subcutaneous mKSA tumor develops an "eclipse" phase such that T cells are no longer capable of being sensitized to reject the tumor; immunocompetence returns after surgical removal of the tumor. Other T cell functions examined in an "eclipsed" host appear not to be affected. We have identified a factor in the ascitic fluid and in the serum that maintains "eclipse" for a long period of time and which also has growth promoting activity Tu-5 (mKSA tissue culture adapted cells) which normally will not grow in the syngeneic BALB/c host will grow progressively given the "factor" from ascitic fluid. Attempts are being made to further characterize this factor biologically and chemically (Henriksen and Law).

b). In contrast to the specific immunodepression observed in "eclipsed" BALB/c hosts bearing mKSA sarcomas, a different situation was encountered in hosts (BALB/c) bearing large plasmacytomas (AdjPC5). Here specific immunity against the TSTA of AdjPC5 was not influenced; on the other hand the immune system (spleen cells) was severely depressed as measured by lymphoproliferative responses to T- and B-cell antigens and reduced SRBC responses Work is in progress to define the mechanisms involved in these different deficiency states.

In the course of investigations on the immunological response of host animals against RCN and lymphocytic leukemias which developed in SJL/J mice neonatally inoculated with D1-MuLV, it was discovered that the lymphoid cell suspension prepared from transplantable RCN showed a marked cytotoxic effect against some other tumor cells in culture. This "killer" effect was not indiscriminatory, but rather selective, and did not require the presence of macrophages. The susceptible target cells included RBL-3, EL4G$^-$, RBL-5, MBL-2, FBL-3 (all of C57BL/6 origin), LPC-1 (BALB/c) and C1.18.4 (C3H). The non-susceptible target cells were D2 (SJL/J), HFL/d-tc (BALB/c), L4946 (AKR), and EδG2 (C57BL/6). Only viable cells of well established D4, RC55, and RC519 of SJL/J RCN were able to show this killer activity. Primary spontaneous RCN or lymphocytic leukemia of SJL/J origin did not show such a killer activity. The killer cell was found to be Fc positive, Thy 1.2 negative, and not affected by pretreatment with anti-Thy 1.2 and complement. Further work is in progress to determine the surface markers on target cells that are recognized by these killer cells. The possibility that the killer activity is exerted by the host cells reacting against RCN is unlikely because SJL/J or C57BL/6 mice immunized with D4 cells did not show cytotoxic activity against RBL-3 or EL4G$^-$ target cells. These data suggest that the transplantable RCN cells were derived from a naturally present, non-T-cell clone that has a cytotoxic effector activity against cells with certain membrane markers. It is postulated that killer cell clones arising spontaneously and destined for immunological surveillance against some (presumably type-C viral, or tumor specific) antigens

present on transformed cells have themselves transformed and become a neoplasm, such as RCN. The fact that, in contrast to the RCN of SJL/J mice, the RCN of BALB/c origin did not show this property is interesting in view of the unique immunological status of SJL/J strain which tends to develop paraproteinemia, dysfunction of humoral antibody production, and impairment of cellular immunity.

Results of studies of tumor immunity employing principally virus-induced leukemia are as follows: Induction of in vivo tumor immunity: the studies of the mechanisms for the induction of in vivo tumor immunity have been hampered by the lack of appropriate methods: on one hand, many of the in vitro assays do not truly reflect in vivo conditions, and on the other hand, it is difficult to separate out different phases of immune response in in vivo experiments for the study. By employing the IRA, we have found a good correlation between the in vitro cytotoxic assay and the in vivo tumor immunity to FBL-3, a Friend leukemia in syngeneic B6 mice. We can further induce the primary and secondary cytotoxic response in vivo by adoptive transfer of lymphocytes. These methods offer us an excellent chance to compare directly the in vitro and in vivo studies and to examine the various phases of immune response in vivo.

Humoral regulation of cell-mediated tumor immunity and host control of tumor growth. By employing the FBL-3 tumor system, we have found that ascitic fluids obtained from various murine tumors and pleural effusions from human patients with malignant tumors can promote the growth of FBL-3 in syngeneic hosts, and to suppress the specific cell-mediated tumor immunity. Thus, a immunosuppressive factor(s) is present in the tumor bearing hosts in mouse and in man. However, if the hosts can recover from the immunosuppressive state, they produce a "counter factor" in the sera that can neutralize the effect of immunosuppressive factor. Thus, hosts also appear to play an important role in the control of tumor growth.

H-2 restriction of T cell-mediated immunity: It has been demonstrated in both virus-infected mice and in tumor systems that T cell-mediated cytotoxic reactions are H-2 restricted: a strict requirement of compatibility at either the K or D ends of H-2 gene complexes among sensitizing cells, target cells and effector cells. These findings, if they were correct, have important implications on tumor immunology. If such strict requirements of histocompatibility genes exist in all tumors, then it will be impossible to prepare a common vaccine for human tumors or find a common tumor antigen for immunodiagnosis. In working with FBL-3 tumor systems, and a 20 hr IUdR release assay, we have found that after prolonged incubation time (20 hr or longer), there is no strict requirement of H-2 homology in mediating T cell killing either in the induction phase or effector phase. Furthermore, our work on the inhibition of the T cell killing by sera and soluble antigen also indicate that tumor associated antigens are entirely different from H-2 antigens i.e. only tumor antigen can inhibit cytotoxic reactions, not H-2 antigen, and only antitumor antibodies can suppress the induction of cytotoxic response to tumor associated antigens, not anti H-2 antibodies.

Early detection of antitumor antibodies in tumor bearing hosts: By employing a sensitive isotopic antiglobulin technique, we can detect the antitumor antibodies at very early stages of tumor growth. The changes in antibody levels are also correlated with the host immune status, however, the pattern of changes may vary in different tumors. These findings indicate that a simple sensitive antibody assay can be used for early diagnosis of cancer and to be used as a monitoring index to evaluate the host immune status.

Evaluation of isotopic cell-mediated cytotoxic assays: We have compared three isotopic assays (^{51}Cr, ^{3}H-proline and $^{125}IUdR$) in measuring cell-mediated cytotoxic reactions against tumor associated antigens and alloantigens. We have found that not only the nature of each assay can affect the results, but also the proper choice of baseline, preparation of target cells and methods used for calculation can also have important effect on the interpretation of the results. These findings indicate the need of standardization of the assays that are used in various laboratories.

Mammalian Cellular Genetics

During this year we have: 1) produced and characterized several valuable chloramphenicol resistant (CAP-r) mutants and fingerprinted their mitochondrial DNA (mt-DNA); 2) repeated, strengthened and extended our findings (reported last year) that CAP-r mt-DNA could "transform" chloramphenicol sensitive cells (CAP-s) to CAP-r; 3) shown that 3 subspecies of Mus, M. musculus, M. m. molossinus and M. caroli can be used to confer the CAP-r phenotype among one another both by cytoplast fusion and by treatment with CAP-r mt-DNA; the cells generated by these maneuvers are being analysed and should provide unequivocal evidence of the mechanism of mitochondrial transformation; 4) suggested that the mechanism by which nuclear DNA from either CAP-s or CAP-r cells is capable of conversion of CAP-s cells to CAP-r is by way of insertion elements that we hypothesize exist among the repeated sequences of the nuclear DNA - a preliminary test of this notion as applied to nuclear genes which sought to measure mutation rates for generation of hypoxanthine-guanosine phosphoribosyl transferase (HGPRTase) deficient mutants (i.e., 6-thio guanine resistant) in CHO cells was, however, inconclusive; 5) acquired through the efforts of Dr. Ho two new techniques that will be crucial to our understanding of the new findings made in the past year - they are nick translation of nanogram quantities of mt-DNA using ^{32}P-nucleotides so that small quantities of restriction fragments might be visualized by autoradiography and similarly, labeling with kinase so that the smaller fragments might be seen.

A major part of our effort has been to try to find methods that will allow the production of mutant and "transformed" cells in yields large enough to extract sufficient mt-DNA for analysis, and use in "transformation" experiments. The most efficient methods of cell production use suspension-adapted strains, however, when we transfered one mutant to such a strain the resultant cells, although produced in large quantity, proved to have few mitochondria and correspondingly little mt-DNA. We have fallen back on roller bottle culture methods more suited for culture of large numbers of fibroblasts. The future success of this project will depend on our ability to de-

velop methods for production of large quantities of the mutant cells and of efficient mt-DNA isolation.

The next step in our analysis of mt-DNA, to be undertaken in the coming year, is the use of heteroduplex mapping (electron microscopy of reannealed mt-DNA molecules from mutants and wild type cells) in order to visualize insertions and deletions. We shall start this endeavor as a collaboration but ultimately will have to develop our own EM facility.

Two new extramural collaborations have begun. One involves the use of functional thyroid tumors and secretory cells from normal animals. This collaboration with Dr. Saverio Ambesi-Impiombato and others at the Istituto di Patoligia Generale at Naples seeks to exploit our important new finding that serum free culture media supplemented by hormones permits maintenance of many significant differentiated functions of various kinds of epithelial cells in culture. The second extramural collaboration is with Dr. G. David Trisler, now in Dr. William Shane's laboratory at AFRRI. Trisler helped with the normally segregating neuron x fibroblast hybrids that were developed in our lab over the past 4-5 years. In addition to corroborating our initial findings of antigens specific to individual neurons (or small groups of them), regionally specific antigens like "theta" have been found to be expressed in a regionally specific manner. The most important application of these neuron hybrid cell lines now appears to be their production of specific brain peptide hormones such as TRF (Thyroid Releasing Factor - demonstrated by two groups in this past year). This new class of hormones has excited much interest of late since they appear to be involved in a wide range of therapeutically significant neural processes from interaction with barbiturate receptors to influences on behavior and mood. The initial discovery of many new members of this new class of hormones may be expected to come from analysis of the synthetic products of these clonal cell lines derived from individual neurons from diverse parts of the nervous system.

Immunochemistry

Chemical and Structural Studies of Antibodies

Mouse Immunoglobulins:

The immunoglobulin secreted by mouse myeloma MOPC 47A is one of a small number of mouse urinary IgA proteins which are two-chain molecules. These molecules consist of a light chain of normal size, disulfide-linked to a shortened heavy (α) chain. We have studied the heavy chain of MOPC 47A in detail. It has a molecular weight of 40,000, contains 6.1% carbohydrate and consists of about 347 aminoacid residues. By a combination of tryptic and cyanogen bromide cleavages, we have obtained the complete aminoacid sequence. Comparison of the data with the sequence of other, non-deleted chains reveals that the 47A chain terminates exactly at the end of the C_H2, so that the deletion in the chain is a single, large deletion consisting of the entire C_H3 domain. This deletion is not due to post-translational modifications. It appears to be non-random, since a number of mouse α chains have been described with the same molecular weight of 47A. Although the genetic event which gives rise to these deleted mouse α heavy chains remains unknown, the most plausible explanation at this moment is that 47A represents a modified C gene, with the

characteristic of another subclass, which could have resulted by an unequal crossing over.

Rabbit Immunoglobulins:

Ample chemical evidence has been given that it is the hypervariable regions that constitute the binding site of the antibody molecules. However, no clear pattern between structure and specificity has been developed. Our aim has been to gain some information on the ligand-binding role of hypervariable region residues. Our present and previous results show that light chain tyrosyl 30 in two different rabbit anti-P-azobenzoate antibodies is involved in the ligand binding. In a third such antibody, tyrosyl 30 is not involved even though it is in fact present. The first hypervariable region which contains tyrosyl 30 differs in sequence in all three light chains. Thus our findings suggest that an antibody site which reacts with a particular structure may be specified by several different sequences and the more or less common presence of a given residue in a particular hypervariable position (e.g. tyrosyl) does not permit assignment of a specific role to that residue.

Although there is no direct evidence that allotypic structures on V_H region have a biologically relevant function, they are detectable on surface membranes-associated Ig of rabbit B lymphocytes and may be associated with some receptors or factors produced by some rabbit T lymphocytes. There is little doubt that sequence differences in the variable region of the heavy chain permit us to distinguish a1, a2, and a3 rabbits serologically. Recently we have obtained evidence that tryptic peptides localized in the variable region of the heavy chain can be isolated with detectable allotypic determinants. Examination of molecular models of three dimensional structures of Igs has provided new insights into the nature and location of the allotypic determinants. It appears that residues 15, 16, and 17 of the heavy chain are in close proximity to residues 84 and 85. These two sets of residues have been strongly implicated as contributors to allotypic determinants.

Antigen Binding Myeloma Proteins

We have previously reported the complete heavy chain variable region sequences from four BALB/c phosphorylcholine binding myeloma proteins as well as partial light chain sequences. These studies have now been extended to induced anti-phosphorylcholine antibodies in other mouse strains. The light chains from three of the BALB/c myeloma proteins have been shown by isoelectric focusing to be present in seventeen other mouse strains tested. Amino acid sequence analysis of these light chains from A/J mice show conclusively the presence of the same framework and complementarity region sequences as are found in the BALB/c myeloma proteins. The heavy chains from the A/J proteins are also identical to those of the BALB/c myelomas. These results establish the germ line nature of the three light chains being analyzed as well as that of the heavy chain and thus begin to elucidate important aspects of the generation of diversity in this system.

A second group of proteins with levan binding activity has been studied to ascertain if the pattern of variation observed among phosphorylcholine binding proteins is applicable to other specificities. Complete heavy chain variable region sequences have been obtained from four of these proteins as well as nearly complete sequences from three of the light chains. In contrast to the phosphorylcholine binding proteins which have light chains from three different subgroups which differ greatly in amino acid sequence the light chains from the anti-levan proteins all belong to the same subgroup and have very few sequence differences between them. The same observation is true for the heavy chains. Whereas both sequence and size differences are found in the complementarity regions of the anti-phosphorylcholine heavy chains the complementarity regions of the anti-levan proteins are all identical. A total of six amino acid differences are found in the frameworks of the four anti-levan heavy chains. Thus, the pattern of variation observed in the anti-phosphorylcholine system cannot be extended as a general pattern to this second specificity.

The group of anti-levan proteins is of additional interest in that members of this group have been shown to possess unique as well as shared or cross-reacting antigenic determinants (idiotypes). The cross reacting idiotypes have been found to be hapten inhibitable and are presumed to be in or near the combining site. By making recombinant molecules in vitro (exchanging light and heavy chains) between one protein positive for a given idiotype and one negative for that determinant, one of the idiotypes studied in detail has been localized to the light chain of the immunoglobulin. A three dimensional model of this protein has been constructed based on data from our previously reported structure of the M603 protein. By analyzing the sequence differences between the light chains in context of the three dimensional structure we have been able to evaluate the probable molecular basis of this idiotype. These studies are being extended to other idiotypes in an attempt to further define the structural basis of idiotypy.

A third group of proteins being analyzed are those with galactan binding specificity. To date nearly complete variable region sequences have been obtained from the light chains of three of these proteins. The variation found is similar to that seen in the anti-levan proteins in that all of the light chains are members of the same subgroup and only very few differences are found between any of the chains. Studies are continuing on the heavy chains from these proteins to permit a comparison with the anti-phosphorylcholine and anti-levan heavy chains. Crystals have been obtained from one of the galactan binding proteins and the three dimensional analysis of this protein is also in progress.

Gene Regulation and Control of Immunoglobulin Structure and Expression

In the last year there has been growing awareness that the allotype (IgC_H) linked immune responses in the mouse could be controlled by a number of different genes acting at different stages in the maturational pathway of the immunoglobulin producing cell. At first many (including ourselves) thought that markers controlling idiotypic differences between two strains could be interpreted to mean a structural gene for a V-region polypeptide had been identified. There are now exceptions, for example, workers in N.

Klinman's laboratory (Univ. Pa.) have shown that markers such as the T15 idiotype (originally described in this laboratory) are expressed at the B-lymphocyte precursor cell level but display phenotypic differences in different strains when antibody levels are used as phenotype. It is now apparent that to demonstrate a V-region structural gene marker a number of specific steps will have to be met including the final and important demonstration through amino acid sequence and three dimensional structure analysis of the polypeptides controlling the idiotypes in the different strains. Nonetheless inbred strain differences in level and expression of idiotype can be used as markers to study the entire pathway of immunoglobulin gene differentiation expression. It is envisioned that three levels are technically accessible: 1) the virgin B cell pool which must be obtained from fetal liver or adult bone marrow, this rapidly becomes diluted; 2) functional B cell pool (which contains a disproportion of V_L-V_H clones amplified through an addition of memory, antigen stimulated cells), or cells from the Gut Associated Lymphoid System (GALT); and 3) the plasma cell pool that can be monitored by serum antibodies.

Finally, many types of markers including idiotypes and even sequences can be ascribed to changes that are probably not directly controlled by germ line gene modifications but by "somatic mutations". There is now abundant evidence that both the germ line gene and somatic theories are correct and that both operate at least to create structural diversity though not necessarily as yet proven functional diversity.

We continue to study the antigenic properties, idiotypes of antigen binding myeloma proteins, both from the point of view of finding potential genetic markers but also to gain insight into the problem Ig gene expression outlined above.

One of the continuing efforts of this laboratory has been to systematize the V-kappa isotypes of the BALB/c mouse. Fortuitously the amino acid sequence of the chains up to the first invariant Trp36 provides a means for typing and identifying genes. The mouse has a great diversity of structures (28 V-kappa isotypes so far identified) which are expressed in the myeloma pool and also in antibodies. It has been found by Weigert and McKean that some antisera to V-kappa chains can be isotype specific. This provides a marker for V-kappa isotype expression, and we are actively engaged in preparing idiotype specific sera to all of the V-kappa isotypes. We have identified a Vk-11 isotype specific antiserum that is found on all of the β2,1F binding myeloma proteins, as well as antibodies of the same specificity. This antiserum will be used to study Vk-11 gene expression in serum, and in the 2 above mentioned B cell pools. We shall extend our knowledge on the diversity of V-k-isotypes by partially sequencing, 25 new kappa chains through Trp36. These data hopefully will define the extent of V-kappa isotypy in the BALB/c mouse. The mouse is an ideal species for studying kappa chain diversity because, for evolutionary reasons not understood the framework regions by different isotypes have many sequence differences. This is in contrast to human V_k in only 4 to 5 framework subgroups have been defined. Thus in man, many V_k isotypes have the same amino terminal framework sequence.

Initiation and Promotion of Plasmacytomas

Specific germ line genes, endogeneous and exogeneous viruses and mutagens (carcinogens) are potential initiating factors. In the mouse there appears to be a strong strain-specific component. Only two inbred strains BALB/c and NZB are thus far known to be susceptible to induction. The identity of genes involved in this process are not yet known. Analysis of the Bailey RI strains that were derived from a cross between the resistant C57BL/6 and susceptible BALB/c indicated the two strains with the BALB/c allotype locus were the most susceptible. This year we have completed a study of two BALB/c congenic strains CB-20 and CAL-20 each of which carries a different allotype. Both of these strains appear to be as susceptible, as BALB/c thus the IgC_H locus from two resistant strains is apparently not linked to a resistance gene. The H-2 locus also does not appear to be associated with a resistance gene.

We will test this year the susceptibility of backcross progeny in a cross between BALB/c and the resistant DBA/2. These mice will be selected for allotype differences and for Tol-1^a gene(s) that govern resistance to tolerization. This specific trait was chosen for study because both BALB/c and NZB are strains that are relatively difficult to tolerize. BALB/c are Tol-1^{bb}, and DBA/2 are Tol-1^{aa}.

The Tol-1^{aa} gene from DBA/2 was backcrossed for three generations onto BALB/c and second generation backcross progeny segregating for Tol-1^a and Tol-1^b will be tested for plasmacytoma susceptibility.

We continue to characterize the conditioning (promoting) effects of the mineral-oil granuloma. Induction of a peritoneal oil granuloma is an essential growth condition for transplantation of primary plasmacytomas in BALB/c mice in the peritoneum. This dependence is rapidily lost during continuous transplantation. This year we described the requirement for an adherent cell in conditioning. In an *in vitro*-assay it was also found that the adherence or interaction of plasmacytoma cells with an adherent cell led to increased DNA synthesis. These studies will be extended to determine the kinetics of this interaction *in vitro*, and to determine if sustained interactions *in vitro* can lead to the *in vitro* adaptation of primary plasmacytomas.

Continued study of conditioning *in vivo* will include studies to determine if T-cells play a role. First, it will be determined if pristane or mineral oil injected nude mice (which lack T-lymphocytes) can, support the growth of primary plasmacytomas. This will be attempted in both BALB/c nu/nu mice and in nu/nu mice of non-BALB/c origin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03200-09 LCBGY

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

A Study of the Various Factors Influencing the Induction, Growth and Repression of Primary and Transplantable Neoplasms (Host and Tumor Cell Variables)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. W. Law	Chief	LCBGY	NCI
OTHER:	E. Appella	Medical Officer (Res.)	LCBGY	NCI
	M. Rogers	Senior Staff Fellow	LCBGY	NCI
	O. Henriksen	Senior Staff Fellow	LCBGY	NCI
	T. Natori	Expert	LCBGY	NCI
	J. L. McCoy	Investigator	LID	NCI
	J. Dean	Investigator	Bionetics	
	R. Ting	Investigator	Biotech	
	K. Takemoto	Virologist	LVD	NIAID
	M. Glaser	Senior Staff Fellow	LCBGY	NCI

COOPERATING UNITS (if any)

Bionetics
Biotech Research Laboratories
NIAID

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

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6.50	2.00	4.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: Major emphasis is placed upon the study of tumor antigens of the transplantation rejection type (TSTA) and of the immune responses they evoke. As a corollary to this study the biologic properties in vitro and in vivo of Histocompatibility (H-2) antigens are under study. Solubilization and methods of purification of both TSTA and H-2 are under investigation with the ultimate purpose of defining these membrane antigens in physico-chemical, biologic and molecular terms.

PHS-6040
(Rev. 10-76)

I. Histocompatibility Antigens

1. Henriksen and Rogers are continuing their studies of H-2 antigen purification ($H\text{-}2^a$ and $H\text{-}2^b$) haplotypes. The most purified $H\text{-}2^a$ antigen obtained following gel filtration, affinity chromatography and PAGE of the papain solubilized material representing a degree of 80-90% purification (600 fold of specific activity) had only limited immunogenicity in congenic mice and did not have tolerogenic activity as was observed for Fr II of G-150 Sephadex fractionation (see annual report of Appella for more specific details).

2. In collaboration with Henriksen we have studied the biologic effects of a non-H2 antigen, the H-Y antigen solubilized by papain. The hope is to eventually compare chemically, in the purified form, H-2 and non-H-2 antigens. It was possible to attain complete tolerance of B/6 females to B/6 male skin grafts through inoculation of solubilized H-Y antigen when inoculated i.p. from day 4 to 10 after birth. Approximately 40% of neonates so inoculated maintained ♂ skin grafts > 100 days but rejected 3rd party grafts. This project has been slowed by our inability to develop a good in vitro assay such as is available for H-2.

3. The role of H-2 antigens in tumor immune responses is being studied as follows:

a). Relation of H-2K and H-2D loci to tumor cell kill. Studies showing that leukemic cell targets are lysed effectively only when effector cells and targets are syngeneic at H-2K and/or H-2D have employed principally the short term ^{51}Cr release assay. Furthermore biologic characterization of "tumor antigens" on the targets used was not accomplished. This study has employed target cells having well-defined TSTA and long term ^{51}Cr release and an IUdR (long term) assay (see Ting for details of results obtained). H-2 restriction may also be studied using SV40 induced-transformants as targets since Glaser now has developed a ^{51}Cr release assay that is specific employing transformed B/6 SV targets. This assay as well as Winn neutralization, both dependent upon T cells as effectors is being used by Glaser to study the kinetics of specific immunity employing solubilized TSTA versus TSTA on intact, viable cells.

b). There is ample evidence in the literature to indicate that the tumor rejection antigens on the cell membrane of chemically induced sarcomas of the mouse are either modified H-2 antigens or represent derepressed H-2 antigens. This project is being pursued in collaboration with Drs. Parmiani and Invernizzi of the National Cancer Institute, Milan, Italy and separately using newly developed sarcomas in this laboratory from pedigreed mice (discussed further under "Tumor Specific Antigens").

II. Tumor Specific Antigens

1. SV40 antigens

The method used for solubilization and attempted purification of H-2 antigens has not been too successful for antigens of the tumor rejection type (TSTA). We reported last year that papain digestion of mKSA membranes probably resulted in extensive degradation since only 2-3% of TSTA activity was recoverable. We now know that an exceedingly small amount of TSTA is present on the membrane of our SV40-induced mKSA neoplasm, the model of choice used for these studies. Newer findings of the characteristics of SV-40 induced TSTA are as follows:

a). In collaboration with Rogers and Appella we have studied the distribution of TSTA in the membrane and nuclear fractions of mKSA sarcoma cells. The preparations obtained had to be free of cross-contamination (that is of TSTA and of T ag); as a consequence membrane marker and nuclear markers were employed. It was found that nuclei were far more effective in protecting BALB/c syngeneic mice against challenge with mKSA than the crude membrane preparation - the membrane containing 5-10 times less TSTA than nuclei. In fact all of the activity of the membrane may be accounted for by contamination with the nuclear T antigen. The majority of TSTA of mKSA cells was consistently found in those fractions that also contained T ag (measured by a quantitative CF assay). Since T ag is not found on the cell membrane, it has been argued that TSTA and T ag are processed differently in the cytoplasm before traveling to the membrane and to the nucleus. We favor the concept, however, that T antigen on the membrane is the effective TSTA and is extremely unstable, with rapid turnover on the membrane. Obviously it is necessary to purify both T ag and TSTA to determine the relationship and the role of the viral DNA in the coding of these antigens.

b). Natori (with Law and Appella) has used another method to solubilize TSTA from the SV40 mKSA membranes - the use of the detergent Nonidet P-40 (NP-40). In contrast to the findings with papain a good recovery (39%) was achieved with solubilization. The soluble antigen was subjected to polyacrylamide-agarose gel filtration (AcA 22 ultrogel column) and tumor rejection activity of the various fractions as well as H-2 and β-2 microglobulin activities were followed. One fraction (V) contained 73% of the activity of the solubilized material whereas only 9% of H-2 activity was recovered in this fraction. The molecular weight range of this fraction was 40,000 - 80,000 daltons. The specific activity of the V fraction remained approximately the same as the crude solubilized material. A reasonable dose-response reactivity was found for the immunogenic fraction V as well as for the solubilized material and these fractions did not affect the growth of the syngeneic Meth-A sarcoma. Of interest is the finding that H-2 activity could be completely separated from TSTA activity through the use of an LcH affinity column.

c). In collaboration with Drs. Rogers, R. Ting (Biotech) and K. Takemoto (NIAID) we studied cross-reactivity patterns of SV-40 transformed cells of several species: man, sheep, orangutan, hamster and sheep using syngeneic mKSA

tumor challenge. All of these transformed cells contained T ag but not infectious SV40. As few as 10^3 intact, viable cells of each of these xenogeneic tissue culture lines provided striking protection. Non SV40 transformed lines representing most of the species did not induce tumor rejection nor did SV40 transformed lines induce immunity against the syngeneic Meth-A sarcoma. The relative amounts of T ag were assayed in all the tissue culture lines and in mKSA. We did not find a good correlation however between T ag concentration and TSTA activity. The crossreactivity of these TSTAs suggest strongly that this antigen as is T ag is coded for by the virus.

d). In collaborative studies with McCoy, Dean and Padarathsingh (Litton Bionetics) and Natori and Henriksen we have used a macrophage migration inhibition assay (agarose droplet) to monitor tumor antigen activity from crude membranes and solubilized products of mKSA cells (SV40 induced). Concentrations as low as 250 picograms protein/ml detected migration inhibition that was specific for the tumor. The suitability of using this assay in detecting activity of tumor antigens during purification procedures is being studied.

e). Meth-A, a chemically induced sarcoma of BALB/c mice, contains strong individually distinct TSTA. Natori in collaboration with Appella and Law has solubilized this antigen from dissociated membranes using NP-40. This TSTA with specific tumor rejection activity has a MW of $\sim$70,000 daltons (in the presence of detergent with an electrophoretic mobility of α-globulin. TSTA was found to separate from H-2. The soluble antigen was extensively purified by a sequence of procedures: gel filtration, lectin affinity chromatography, column electrophoresis. The specific activity of TSTA increased during the purification procedure and in the final step used, PAGE, only 3 major bands were detected. A very small amount of purified antigen 4 μg provided greater than 50% specific tumor inhibition in the syngeneic system and a good dose-response reactivity was attained.

An additional antigenic specificity was identified on Meth-A membranes through the preparation of a xenogeneic antiserum (in rabbits) using the partially purified TSTA as antigen and extensively absorbing the antiserum in vivo. This antigen designated, TSSA, was further purified with retention of both tumor rejection and TSSA activity (assayed by a radioimmunoassay). The TSSA antigen was shown to have a MW of 60,000 daltons and on electrophoretic mobility of α-globulin. Studies are in progress to determine the relationship between TSTA and TSSA.

f). Rogers (in collaboration with Law and Appella) has continued the study of TSTA of lymphocytic neoplasms induced by FMR viruses (but specifically RBL-5). The role of the viral structural entities, gp70 and p30, functioning as TSTA has been studied. These proteins, as well as p15, have been identified on cell surfaces by cytotoxic assays employing xenogeneic antisera and their role in prevention of leukemia (erythroblastosis?) and tumor rejection (in the rat principally) has been emphasized by several investigators.

TSTA solubilized from RBL-5 membranes were extensively digested with papain; this solubilized material retained specific tumor rejection activity and inhibitory activity in in vitro cytotoxic assays. More than 90% of the activity of p30 and gp70 was destroyed. Also, purified p30 and gp70 (obtained from Dr. S. Oroszlan) had no appreciable influence when used as immunogens in tumor rejection or as inhibitors of specific cell mediated cytotoxicity (in collaboration with Dr. C. C. Ting) we have not yet been able to study other translational products of the C-type genome such as p10, 15 and 12.

In extensions of this work Drs. Rogers and Prat have introduced a new technique of using a Stansted cell disruptor to obtain purified cell membranes. Solubilization was achieved by Na deoxycholate (DOC) followed by chromatography on an AcA-34 agarose-acrylamide gel column. Nine separate fractions were assayed for tumor rejection activity against RBL-5 (and MCA-10, Meth-A and mKSA as specificity controls). The findings of significance were:

1). Fractions 1 (aggregated void volume) 2, 3 and 4 contained the major portion of tumor rejection activity. As low a concentration as 1 μg for example of fractions 2 and 3 provided greater than 50% tumor inhibition. A reasonable dose-response activity was obtained in the dosage range 0.5 μg to 50 μg.

2). Fraction 5, containing both H-2 and gp70 activity and the combined fractions 6 and 7 (containing major gp70 activity) provided only minimal tumor rejection activity.

3). Only fraction 8 material inhibited the cytotoxic complement-dependent activity of our syngeneic anti-crude membrane serum. This antiserum has been found capable of lysing purified Rauscher virus (S. Oroszlan). Since only antisera directed against gp70 and p15E are capable of lysing virus, fraction 8, therefore contains some p15E antigen. p30 has not been detected in any of the fractions. The bulk of the evidence obtained to date therefore favors the concept that there exists on MuLV-induced leukemic cells specific TSTA entities free of viral structural proteins.

g). Relation of tumor specific antigens to H-2 antigen

The role of H-2 antigens either in a modified form or existing as derepressed antigens in tumor rejection has been reported in the literature.

1). Meth-A soluble antigens are separated by chromatography and attempts to detect alien H-2 specificities on Meth-A cells has not been successful. It appears that, for this sarcoma, TSTA are distinct from H-2.

2). In collaboration with Dr. Parmiani (NCI, Milan) Rogers and Prat have completed some preliminary investigation using a BALB/c chemically induced sarcoma having alien $H\text{-}2K^{k}$ specificities that appear to be the tumor rejection antigen (TSTA). It has been confirmed using papain solubilized antigens from this neoplasm C-1 (1) that a) the alien specificities do exist

and are not artifacts (as determined by inhibition of cytotoxicity and a radioimmunoassay) and b) that the normal H-2D specificities do exist on the membranes and both are located in the same molecules purified from the membranes. This investigation will proceed but preparations of the membranes from C-1 (1) for adequate yield need to be done in this laboratory.

3). We have initiated a study using pedigreed BALB/c mice to induce a series (6) of sarcomas by methylcholanthrene. Individual TSTA have been identified (no cross reactivity) in all neoplasms. Each neoplasm is being studied for the existence of alien specificities. If detected by in vivo assays (through the use of F_1 hybrid recipients) membranes will be solubilized and specific H-2 specificities determined. The precise role of these can then be determined through fractionation procedures of the solubilized products.

III. Immune Reactions of the Tumor Bearing Host

a). The host bearing a large > 25mm, subcutaneous mKSA tumor develops an "eclipse" phase such that T cells are no longer capable of being sensitized to reject the tumor; immunocompetence returns after surgical removal of the tumor. Other T cell functions examined in an "eclipsed" host appear not to be affected. We have identified a factor in the ascitic fluid and in the serum that maintains "eclipse" for a long period of time and which also has growth promoting activity Tu-5 (mKSA tissue culture adapted cells) which normally will not grow in the syngeneic BALB/c host will grow progressively given the "factor" from ascitic fluid. Attempts are being made to further characterize this factor biologically and chemically (Henriksen and Law).

b). In contrast to the specific immunodepression observed in "eclipsed" BALB/c hosts bearing mKSA sarcomas, a different situation was encountered in hosts (BALB/c) bearing large plasmacytomas (AdjPC5). Here specific immunity against the TSTA of AdjPC5 was not influenced; on the other hand the immune system (spleen cells) was severely depressed as measured by lymphoproliferative responses to T- and B-cell antigens and reduced SRBC responses (Padarathsingh, Dean and Law). Work is in progress to define the mechanisms involved in these different deficiency states.

Significance for Cancer Research:

Characterization of tumor antigens of the rejection type is a necessary prerequisite for understanding the mechanisms of immune surveillance, tumor inhibition and facilitation. In addition the role of these membrane bound components in the actual initiation and maintenance of malignancy may be studied.

As a basis for any study of membrane bound antigens it is necessary to study the nature of histocompatibility antigens and their relationship to tumor antigens. Thus our emphasis is on parallel studies of H-2 antigens in order to provide a basis for understanding tumor antigens.

Objective 3; Approaches 4,5.

Publications:

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Appella, E., Rogers, M. J. and Law, L. W.: Biochemical and biological properties of histocompatibility antigens and tumor specific transplantation antigens. In Elsevier/North-Holland (Eds.): Proceedings of the International Congress on HLA Systems - New Aspects, Bergamo, Italy, 1976 (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03222-09 LCBGY

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Genetic and Biochemical Mechanisms Controlling Protein Synthesis in Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the control mechanisms important in regulating protein synthesis in normal and malignant mammalian cells.

A. Transcription of and translation by transfer RNA (tRNA) is being studied by comparing isoaccepting tRNA chromatographic profiles of normal tissues and tumors. tRNA from normal organs of mice and rats, primarily liver and brain, is being compared with tRNA from many different spontaneous and transplantable mouse and rat tumors, particularly mouse plasmacytomas and rat hepatomas. Of special interest is the origin, structure and function of tumor specific phenylalanyl-tRNA found only in certain mouse and rat tumors.

B. The synthesis and structure of IgA half molecules, mouse IgA myeloma proteins made up of one light chain disulfide bonded to one unusually short heavy chain, is also of major interest, particularly to determine if these molecules represent a normal IgA subclass or products of aberrant or incomplete synthesis at the DNA, RNA or protein synthetic level.

Methods Employed:

A. Transfer RNA from Mouse and Rat Tumors

The variations in isoaccepting tRNAs among tumors and normal tissues are being studied using: 1) reversed phase, high pressure liquid chromatography, 2) polynucleotide and triplet directed ribosome binding, 3) cloning in tissue culture, 4) utilization in cell free protein synthesis, and 5) RNA sequencing techniques.

B. IgA Half Molecule Mouse Myeloma Proteins

These unusually small IgA immunoglobulins are being studied immunochemically using: 1) homologous and heterologous antiserums in agar gel double diffusion and hemagglutination inhibition, 2) polyacrylamide gradient gel electrophoresis, 3) exo- and endopeptidase digestion, 4) cloning of mutant lines of tissue culture adapted plasmacytomas, and 5) assay of normal mouse tissue extracts for localization of IgA half molecules in normal mice.

Major Findings:

A. Transfer RNA

1. Phenylalanyl-tRNA from Various Tumors

We have determined that an extra, tumor-specific "Phe-tRNA$_1$" isolated from rat hepatoma 7777 differs from normal "Phe-tRNA$_2$" by not having the modified base "Y" which is characteristic of most eukaryotic Phe-tRNA. In order to determine how wide spread this "Y absence" is and how general a property this is for tumors we surveyed 22 rat neoplasms of different cell types, 20 mouse tumors of various origins, 11 mouse plasmacytomas, and 4 normal organs of rats and mice for the presence or absence of this early-eluting, Y-minus Phe-tRNA. Normal tissues never contained this Phe-tRNA even when extracts of embryonal or fetal liver were examined. Such a peak could be artificially generated from normal Phe-tRNA if buffering during tRNA preparation was inadequate. This artificial Y-minus Phe-tRNA is easily confused with the tumor-specific peak, but we developed a test using 3-methyl-2-benzothiazolinone hydrazone (MBTH) which reacts only with tRNA from which an entire base, such as Y, has been excised. 15/22 rat tumors had peaks of tumor specific Phe-tRNA (not reacting with MBTH); 12/21 mouse tumors surveyed had an MBTH unreactive tumor peak of Phe-tRNA$_1$, and in addition, none of the 11 lines of BALB/c plasmacytomas contained such a molecule. There was a significant difference between the tumor peaks of Phe-tRNA$_1$ in mice and rats. Those mouse tumors that were positive for this marker had a nearly complete replacement of their Phe-tRNA with this Y-minus tRNA, whereas the proportion of Y-minus Phe-tRNA in rat tumors never exceeded 20% of the total Phe-tRNA.

2. Mouse Plasmacytoma tRNA Comparisons

Eleven lines of BALB/c plasma cell tumors were selected for tRNA studies because of the nature of the immunoglobulin they secreted. Enough of the structure of the secreted Igs is known so that tRNA from tumors with small differences in hypervariable regions, with identical idiotypes, with different heavy chain classes, and non secreting tumors with their parent secreting line could be selected for comparison. This work is now virtually complete for all 20 natural amino acids, and the patterns can be described as variable or constant depending on which amino acid is being considered. The most variable among the tRNA profiles of the tumors studied also included peaks of tRNA or groups of peaks that seemed to be abundant in nearly all plasmacytomas but virtually nonexistant in normal mouse liver. This group included Asn, Ser, Trp, and Val-tRNA. Another group includes aminoacyl-tRNAs with patterns showing tremendous variability among the tumors studied although a characteristic tumor pattern was not evident in all tumors: Glu, His, Lys, Thr, and Tyr. Two amino acids have patterns not varying much among most tumors, but significant pattern variations occur for a few tumors only: Pro and Glu. The final group of nearly half of the natural amino acids has tRNA patterns amazingly similar among all eleven tumors and normal liver: Ala, Arg, Asp, Cys, Gln, Gly, Leu, Met, and Phe.

B. IgA Half Molecules (IgA_h)

Homologous IgA_h specific antiserums were developed to search for this type of molecule in normal mouse tissues. The search was successful, but only in normal BALB/c gut contents was IgA_h consistently identified. This protein was found only in a Sephadex G-100 fraction also containing albumin, so it appears that IgA_h molecules represent a normal molecule of the size of the IgA_h myeloma proteins, 65,000 MW.

Significance:

A. Transfer RNA

Our survey of the RPC-5 column profiles for all 20 amino acids for 11 carefully selected mouse plasma cell tumors is by far the most extensive and most complete study of its type. Chromatographic tRNA comparison studies by other investigators have been put into context by our results and some of their conclusions shown to be premature due to the use of too small a series of tumors and tissues. The tremendous variation in tRNA patterns from tumor to tumor, for the aminoacyl-tRNAs specified above, makes correlation with other tumor characteristics very difficult. For example, two immunoglobulin hypervariable region amino acid substitutions appeared to be accompanied by a special peak of the appropriate aminoacyl-tRNA, two others did not. We did show that tumors closely related by clonal origin are likely to have similar tRNA profiles. This suggests that the expression of tRNA genes is fixed rather early in the carcinogenic process, and that most later mutations such as turning off protein secretion has little effect on the expression of tRNAs. Thus, whereas normal cells like reticulocytes and silk worm silk glands seem

to turn on new genes or amplify the products of old ones in response to demands of major tRNAs, these tumor cells may not do so.

tRNA pattern characteristics of nearly all of the tumors were apparent for several amino acids. Preliminary evidence indicates that many of these tumor-like patterns are also found in tRNA from mouse brain, but not with liver, spleen or kidney. It is likely that most of these pattern peculiarities reflect an intracellular enzymatic environment that is responsible for the many post-transcriptional modifications of the tRNAs in question. The tumor specific Phe-tRNA, on the other hand, does not have a counterpart in any normal adult or fetal tissue yet tested and its presence only in tumors remains very intriguing but its functional significance remains to be elucidated.

B. IgA Half Molecules (IgA_h)

An IgA half molecule was recently reported in a case of human multiple myeloma with many characteristics of the mouse IgA_h being studied here. Although not recognized as a normal IgA subclass in man or mouse, IgA_h seems quite likely to be one in mice.

Proposed Course:

A. We feel that the large number of chromatographic profiles already generated for comparison adequately completes the study of tRNA distribution in tumors. Two aspects are worthy of follow-up: 1) to determine whether the unusual tRNAs in tumors function normally as adaptors in protein synthesis by triplet binding studies and cell free protein synthesis; and 2) to see if the structure of tumor-specific Phe-tRNA in mouse and rat differs by more than just the Y base which will tell whether these tRNAs represent new gene activations or imcomplete post transcriptional modifications. These relatively limited, collaborative studies of translation and tRNA primary structure should terminate our study of tRNA.

B. We expect to confirm that IgA_h represents a second mouse IgA subclass by 1) doing comparative studies of the C-terminal peptide of all 6 IgA_h "deleted" heavy chains; 2) finding normal IgA_h molecules in other mouse strains, 3) testing for antibody activity, and 4) testing for low sensitivity to purified protease digestion or digestion by gut contents.

Relationship:

Objective - 3

Approach - 1

Publications:

J. Frederic Mushinski and Miranda M. Dungan: IgA Half molecules. II. Genetic variants of IgA detected in normal mouse intestinal contents. J. Immunol., 117: 1668-1675, 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03229-09 LCBGY

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
I. Chemical structure of immunoglobulin molecules, allotypes and human histocompatibility antigens. II. Biochemical characterization of soluble murine H-2 antigens, tumor specific antigens and epoxide hydrase.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.25	4.50	0.75

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chemical and Structural Studies of Antibodies and Histocompatibility Antigens. It is the purpose of this project to study the chemistry and structure of antibodies and histocompatibility antigens. Antibodies of different specificities and allotype are purified. The chemial sequence of both the light and heavy chains is obtained in order to gain some insight into the nature of antibody specificity and the generation of diversity. Improvements in the isolation methods of histocompatibility antigens and tumor antigens are currently being pursued. The characterization of the chemical structure of these proteins is being approached by micro methods and the relationship of the amino acid sequence to immunoglobulin structure or other known structures is being analyzed. Epoxide hydrase plays a critical role in chemical-induced mutagenesis and carcinogenesis. Chemical modification studies have been undertaken with the goal of identifying aminoacid residues which are essential for catalytic function.

PHS-6040
(Rev. 10-76)

1. Mouse Immunoglobulins:

The immunoglobulin secreted by mouse myeloma MOPC 47A is one of a small number of mouse urinary IgA proteins which are two-chain molecules. These molecules consist of a light chain of normal size, disulfide-linked to a shortened heavy (α) chain. We have studied the heavy chain of MOPC 47A in detail. It has a molecular weight of 40,000, contains 6.1% carbohydrate and consists of about 347 aminoacid residues. By a combination of tryptic and cyanogen bromide cleavages, we have obtained the complete aminoacid sequence. Comparison of the data with the sequence of other, non-deleted chains reveals that the 47A chain terminates exactly at the end of the C_H2, so that the deletion in the chain is a single, large deletion consisting of the entire C_H3 domain. This deletion is not due to post-translational modifications. It appears to be non-random, since a number of mouse α chains have been described with the same molecular weight of 47A. Although the genetic event which gives rise to these deleted mouse α heavy chains remains unknown, the most plausible explanation at this moment is that 47A represents a modified C gene, with the characteristic of another subclass, which could have resulted by an unequal crossing over.

2. Rabbit Immunoglobulins:

Ample chemical evidence has been given that it is the hypervariable regions that constitute the binding site of the antibody molecules. However, no clear pattern between structure and specificity has been developed. Our aim has been to gain some information on the ligand-binding role of hypervariable region residues. Our present and previous results show that light chain tyrosyl 30 in two different rabbit anti-P-azobenzoate antibodies is involved in the ligand binding. In a third such antibody, tyrosyl 30 is not involved even though it is in fact present. The first hypervariable region which contains tyrosyl 30 differs in sequence in all three light chains. Thus our findings suggest that an antibody site which reacts with a particular structure may be specified by several different sequences and the more or less common presence of a given residue in a particular hypervariable position (e.g. tyrosyl) does not permit assignment of a specific role to that residue.

Although there is no direct evidence that allotypic structures on V_H region have a biologically relevant function, they are detectable on surface membranes-associated Ig of rabbit B lymphocytes and may be associated with some receptors or factors produced by some rabbit T lymphocytes. There is little doubt that sequence differences in the variable region of the heavy chain permit us to distinguish a1, a2, and a3 rabbits serologically. Recently we have obtained evidence that tryptic peptides localized in the variable region of the heavy chain can be isolated with detectable allotypic determinants. Examination of molecular models of three dimensional structures of Igs has provided new insights into the nature and location of the allotypic determinants. It appears that residues 15, 16, and 17 of the heavy chain are in close proximity to residues 84 and 85. These two sets of residues have been strongly implicated as contributors to allotypic determinants.

3. Histocompatibility and Tumor Specific Transplantation Antigens:

Intensive work has been devoted to the purification and chemical characterization of H-2 antigens. Membranes have been prepared from liver cells by differential centrifugation according to established procedures. The membranes are digested by limited papain which solubilizes H-2. The solubilized H-2 is purified by DEAE, gel filtration, affinity chromatography on concanavalin, and isoelectric focusing. By inhibition of binding of radiolabeled antigen, this material has been shown to be about 80% pure. A single peak of about 40,000 could be obtained after full reduction and alkylation and filtration on G-100 Sephadex column. This might represent the purified heavy chain of the H-2 molecules. A chemical analysis has shown a blocked amino-terminal. Peptide analysis is now in progress and a comparison with the chemical structure of HLA molecules should reveal new insights into the evolutionary relationship of these molecules and the immunoglobulins.

Tumor specific transplantation antigens (TSTAs) are widely recognized phenotypic characteristics of neoplastic cells. Their existence provides the major rationale for an immunological approach to cancer therapy. We have been interested in the SV40 system in the mouse, as a model for studying TSTA. A protein of the early transcribing region of SV40 (cistron A) contains the information for SV40 DNA replication and for both the initiation and maintenance of certain aspects of the transformed state. Three distinct antigenicities have been shown to be specified by the SV40 early genome, the tumor or T antigen, U antigen and TSTA. Both T antigen and TSTA appear to be virus specific. Estimates of the size of the early region DNA indicate that it can code for proteins of molecular weight not greater than 100,000. Since the molecular weight of T antigen has been shown to be around 94,000, it has been suggested that T antigen, TSTA and U antigens are encoded by the same DNA sequence. We have obtained in vivo data which show that most of the TSTA activity is found along with T antigen in the nuclei. This finding is consistent with the idea that T and TSTA are part of the same protein molecule; whether the A protein carries antigenic sites for TSTA as well as T antigen is not proven at this time. The A protein may undergo cleavage and/or modification before localization in the cell membranes where it may then interact with other membrane molecules. We are at the present investigating these possibilities.

Chemically induced tumors also contain TSTAs. These antigens are unique, in that each tumor induced by the same chemical in the same inbred strain has its own antigen. The genetic origin of these new antigens is not known. We have started to purify such antigens from a methylcholanthrene induced tumor (Meth-A) and hope to provide a more precise answer as to their origin.

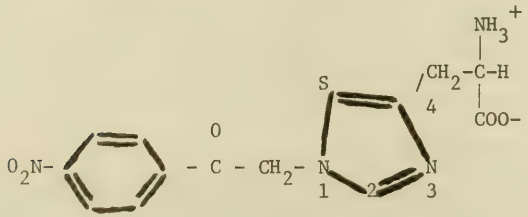
4. Chemically Modification of Epoxide Hydrase:

Epoxide hydrase, and enzyme localized in the liver microsomes, plays a critical role in chemical-induced mutagenesis and carcinogenesis. In an effort to investigate the mechanism of action of this enzyme, chemical modification studies have been undertaken with the goal of identifying amino acid residues which are essential for catalytic function.

2-Bromo-4'-nitroacetophenome (Br-4-NAP) is a potent and specific inhibitor of epoxide hydrase. The enzyme is rapidly and irreversibly inactivated by greater than 10-fold molar excesses of the reagent, displaying pseudo-first order kinetics. A pH-rate of inactivator profile shows that the rate of inactivation is controlled by a group on the enzyme with an apparent pk of 7.5. The enzyme is protected from inactivation by the presence of substrate and incorporation studies with ^{14}C labelled Br-4-NAP show the stoichiometry of the reaction to be slightly greater than 1 mole of reagent/mole of protein when the enzyme is 95% inactivated.

Amino acid analysis of native and Br-4-NAP modified epoxide hydrase indicates the loss of approximately 1 histidyl residue, although methionine could not be ruled out as a possible site of alkylation. Methionine was eliminated as the site of alkylation based on further experiments. Performic acid oxidation and amino acid analysis showed no difference in methionine content between modified and native epoxide hydrase. In addition, no homoserine or homoserine lactone could be detected in the acid hydrolysates of the modified enzyme. Homoserine or homoserine lactone would be expected decomposition products resulting from the acid hydrolysis of a phenacyl sulfonium salt. Finally ^{14}C labelled Br-4-NAP modified enzyme was not appreciably dealkylated by a large excess of α-mercaptoethanol. Phenacylmethionine sulfonium salts are rapidly dealkylated by thiols.

Although no N-alkylated histidine derivative was detectable in the amino acid analysis of the modified enzyme, derivatives of this type are known to be stable during acid hydrolysis. A sample of ^{14}C labelled epoxide hydrase (1.1 moles Br-4-NAP/mole of protein) was hydrolysed under the conditions for amino acid analysis. The hydrolysate was separated into its constituent amino acids by high voltage paper electrophoresis and a radioautogram prepared. The radioautogram revealed the presence of a single radioactive spot. The identity of this radioactive amino acid was confirmed as an N-alkylated derivative of histidine by comparison of its electrophoretic and chromatographic mobility with authentic samples of the isomeric 1,4 and 1,5 p-nitrophenacyl histidine derivatives. The N-alkylated histidine derivative formed as the result of the inactivation of epoxide hydrase by Br-4-NAP was further identified as 1-(p-nitrophenacyl)-4-histidine and its structure is shown below.



In order to identify a partial amino acid sequence containing the N-alkylated histidine derivative, peptide mapping studies have been undertaken. A 9mg sample of the modified epoxide hydrase (1.1 moles of ^{14}C-Br-4-NAP/mole of protein) was succinylated with succinic anhydride to mask the lysyl residues. The protein was reduced and carboxymethylated, and digested with trypsin. Gel filtration chromatography or Sephadex G-75 revealed the presence of two ^{14}C- labelled peptides, a small peptide MW ~ 2000 and a larger peptide which eluted with the void volume. The presence of two ^{14}C labelled peptides is probably the result of incomplete digestion by trypsin and the smaller peptide is probably a cleavage product of the partially digested larger peptide. Experiments are currently in progress to purify and sequence both of these peptides.

5. Studies on Alien H-2 Specificities:

Drs. Prat and Rogers have examined the probability of existence in a chemically induced leukemia of SJL/J mice (H-2^s haplotype) of alien H-2 specificities detected by complement-mediated lysis. This anomalous reactivity was found to be due to previously undetected public specificities that were also found in normal cells of the H-2^s haplotype.

Publications:

Natori, T., Tanagaki, N., Pressman, D., Henriksen, O., Appella, E. and Law, L. W.: The component fragments obtained by acid dissociation of papain-solubilization H-2 molecules. Immunogenetics 3: 35-48, 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04833-09 LCBGY

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biological Studies of Various Normal and Virus-Infected Malignant Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. A. Wivel	Head, Ultrastructural Biol.	LCBGY NCI
	S. S. Yang	Chemist	LCBGY NCI

COOPERATING UNITS (if any)

M. Potter LCBGY NCI
R. Gantt LB NCI
P. Pitha Johns Hopkins University School of Medicine

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Ultrastructural Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or their precursors may be the transforming agent. A variety of mouse model systems are used, including plasma cell tumors, mammary tumors, and neuroblastomas. Current projects include: 1) characterization of the properties of intracisternal A particles; 2) effects of interferon on the assembly of murine oncornaviruses; 3) image analysis of virus particles using a computerized scanning electron microscope system; 4) correlation of cell ultrastructure with immunoglobulin synthesis in Abelson virus-induced plasma cell tumors and lymphosarcomas.

PHS-6040
(Rev. 10-76)

Major Findings:

1. Intracisternal A particles. In a continuing study of mouse intracisternal A particles (IAP), it has been established in a collaborative study with Dr. Raymond Gantt, that such particles apparently have a number of tRNA methylases. It has been previously shown that IAP have a number of biochemical markers which are characteristic of RNA tumor viruses. Appropriate experiments indicate that the tRNA methylase specific activity is highest in purified preparations of IAP and that it is bound within the particles rather than simply being associated with the outer surface. The latter finding is documented by the fact that detergent treatment of IAP with 1% Triton does not remove tRNA methylase activity, although this concentration of detergent is sufficient to remove the outer shell of IAP. Current efforts are directed toward a comparison of the IAP-associated tRNA methylases with those of the host cell. The results should give a strong indication about whether these methylases are coded for by the particle genome or whether they are simply cell methylases which are incorporated during the process of replication in a manner analogous to incorporation of cell actin by some budding enveloped viruses.

2. Interferon treatment of murine oncornaviruses. Continuing studies with Dr. Paula Pitha have added to our knowledge about the effects of interferon on in vitro AKR virus infection. There appears to be a differential effect exhibited by interferon which is dependent on the host cell used to replicate the virus. When AKR-L1 cells are infected with AKR virus and then treated with interferon, there is a block in virus assembly with arrest at the budding stage. Thus there is a significant inhibition of virus release from the cell, and the virus titers in the tissue culture medium are reduced accordingly. When SC-1 cells, derived from a feral mouse and lacking FV-1 restriction, are infected with AKR virus and treated with interferon, the block in virus assembly does not occur at the budding stage, but whole virions remain trapped at the plasma membrane of the cell. Again the virus titer in the tissue culture medium is reduced. Trypsinization of the infected, interferon-treated SC-1 cells does not appear to release significant amounts of the cell-associated virus. However, the results seen with both AKR and SC-1 cells tend to indicate that the mechanism of interferon action is associated with membrane phenomena that are critical to a late stage of virus assembly and release. Since interferon could inhibit the translation of a virus protein necessary for assembly, current efforts are directed toward the characterization of interferon-treated virions to determine if there are intrinsic polypeptides missing.

3. Use of scanning electron microscopy plus a computer for image analysis of virus particles. As an adjunct to the aforementioned interferon studies, a new system for analysis of C-type viruses has been successfully employed. Through the use of the scanning electron microscope (SEM), it is possible to obtain quantitative counts of virus particles distributed over much larger surface areas of the cell than could possibly be done by transmission electron microscopy. Specific confirmation of the SEM results is provided by taking

different portions of the same cell sample, doing virus titers of the infected cells, and doing thin-section identification of the virus by transmission electron microscopy. Since the SEM generates an image in a step-by-step manner, the image can be reduced to a matrix of picture points. When the electron beam is controlled by the digital sweep generator of the computer, the dwell-time on a given image point can be precisely controlled, which facilitates the simultaneous collection of all signals of interest at a given picture point. In the case of the C-type viruses, the particles are analyzed three-dimensionally by measuring 8 separate diameters. The ratio of the minimum diameter to the maximum diameter, (the aspect ratio), is then determined, and in the case of true spheres this value is approximately one.

In the instance of the interferon-treated C-type viruses, there is an accumulation of either whole virus or viral buds at the cell surface. The data from the SEM-computer system confirm the situation in quantitative statistically significant terms. In a representative experiment, the infected control cells showed 2.67 particles per field of cell surface, while in the interferon-treated infected cells there were 10.67 particles per field of cell surface. It seems apparent that this automated approach is both feasible and accurate, and it has been confirmed by manually counting the fields instead of using the computer.

4. Ultrastructure of Abelson virus-induced tumors. In a continuing collaboration with Dr. Michael Potter, we have been studying the properties of these tumors in a tissue culture system. When the tumors were studied in animal transplant it was determined that there was a direct correlation between the presence of secretory organelles (plasmacytoid and plasma cells) and the ability to secrete immunoglobulins or IA antigen. When these tumors are grown in vitro there is some evidence of dedifferentiation, i.e., the proportion of plasmacytoid cells decreases and the proportion of small lymphocytes increases; there is a related decrease in secretory capacity. Of interest is the fact that one granulocytic cell tumor has been induced by Abelson virus. This is predominantly of a single cell type and has remained essentially unchanged in tissue culture. The existence of both lymphocytic and granulocytic tumors suggests that a primitive hematopoietic stem cell with a totipotential capacity for differentiation can be the target for malignant transformation by Abelson virus.

Publications:

Wivel, N. A. and Yang, S. S.: Murine Neuroblastoma Clones with Varying Degrees of C-type Virus Expression. Int. J. of Cancer 18:236-242, 1976.

Yang, S. S. and Wivel, N. A.: Physicochemical Analysis of the Deoxyribonucleic Acid Product of Murine Intracisternal A Particle RNA-Directed DNA polymerase. Biochim et Biophys Acta 447:167-174, 1976.

Malech, H. L. and Wivel, N. A.: Transfer of Murine Intracisternal A Particle Phenotype in Chloramphenicol-Resistant Cytoplasts. Cell 9:383-391, 1976

Potter, M., Premkumar, E., and Wivel, N. A.: Immunoglobulin Production by Lymphosarcomas Induced by Abelson Virus in Mice. In Sanford, K. K. (Ed.): Third Decennial Review Conference on Cell, Tissue and Organ Culture. NCI Monograph Series. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 04834-02 LCBGY

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Viral gene expression in normal tissues and in virus- or chemically induced neoplasms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. S. Yang	Chemist	LCBGY	NCI

COOPERATING UNITS (if any)

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H. M. Malech	Research Associate, Ultrastructural Biology Section LCBGY, NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	.50	1.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major thrust of this study is to elucidate the basis of neoplastic transformation of normal tissues. Two experimental model systems were used in this project: (1) a rat hepatoma induced by the chemical carcinogen N,N-2,7-fluorenylene bis-2,2,2-trifluoroacetamide, which has been adapted to tissue culture (HTC cells); and (2) two intracisternal A particle (IAP) containing neoplasms, a plasma cell tumor (MOPC-104E) and differentiated neuroblastoma, also adapted to tissue culture (cell lines N-4, N-18, and NB-2A). Multidisciplinary approaches were required in this investigation. They involve tissue culture, biochemical and biophysical analyses, cytogenetics, immunology and tumor biology. Areas with special emphasis of research effort are: (1) extensive characterizations morphologically biologically, biochemically and immunologically of KiMSV(RHHV), a transforming viral complex successfully rescued out of a co-culture of rat hepatoma tissue culture cells (HTC-H1) with K-NRK cells; (2) regulation of viral gene (GAG for gs antigens and ENV for envelope antigen) expressions; (3) the oncogenic potential of KiMSV(RHHV); (4) virus infection and the development of chromosome abnormalities during the process of neoplastic transformation, and (5) integrations of viral genome and its localization on host cell chromosome(s).

Major Findings:

I. Characterizations of a novel transforming virus, KiMSV(RHHV).

Although much work has been done in the areas of tumor biology the basis of how tissues come to lose their normal cellular control mechanism over metabolism and develop into the neoplastic state remains to be elucidated. Our major effort has been concentrated toward a better understanding of neoplastic transformation by adopting as an experimental model system the chemically induced rat hepatoma, in tissue culture, called HTC cells. During the past year extensive efforts in genome rescue experiments resulted in the isolation of a rat heptoma virus pseudotype Kirsten sarcoma virus, KiMSV(RHHV), when HTC-H1 cells were co-cultivated with Kirsten sarcoma virus transformed non-producer rat kidney cells (K-NRK) (J. Gen. Virology 1976, 31, 239-243). As an on-going project characterizations biologically, morphologically, biochemically and immunologically of KiMSV(RHHV) have now been completed. It has a C-type virus ultrastructure. It is strictly rat tropic in host range. The helper virus, RHHV, originates from the hepatoma cells as suggested by the mode of its rescue and by its host specificity. KiMSV(RHHV) sediments isopycnically in a linear sucrose gradient at 1.145-1.155 g/cm^3, a lesser density than that of the mouse C-type tumor viruses (1.160 g/cm^3). It also possesses the complements of RNA and reverse transcriptase. Results obtained by indirect fluorescent-antibody assays and neutralization bioassays indicate that KiMSV(RHHV) exhibits a major rat leukemia virus (RaLV) specific antigen (GP 70, glycoprotein of 70,000 mol. wt.) and an inter-mammalian group specific gs-3 (P30, 30,000 mol. wt. protein) antigen.

II. Regulation of viral gene expressions.

The major viral proteins associated with KiMSV(RHHV), labelled in either ^{35}S-methionine or ^{3}H-amino acids, were analyzed by 5-30% gradient polyacrylamide gel electrophoresis. They consist of the GAG gene products peaking at the 70,000 mol. wt., the 30,000 mol. wt. (P30), and an 8-10,000 mol. wt. (P10) area. A major peak of radioactivity was observed at the 200,000 mol. wt. area; it is a glycosylated protein as discussed below. Incorporation studies with ^{3}H-glucosamine and ^{14}C-galactosamine showed that these precursors for glycoprotein were incorporated into a large molecule fraction of 200,000 mol. wt. and also a protein fraction of 70,000 mol. wt. Origin of the high mol. wt. glycoprotein 200,000, whether from cell or from virus, has yet to be determined. The latter fraction is the ENV gene product, gp 69-70. Currently we are concentrating on the regulation of synthesis and assembly of these major viral gene products by radioactive pulse-chase experiments using virus infected cells. The results of these experiments are compared to those obtained in a cell-free protein synthesis system with transcribed mRNA dictated by viral cDNA transcribed from the 5'OH end of the viral genome using KiMSV-(RHHV) reverse transcriptase. By these studies we hope to resolve the order of GAG and ENV genes in the viral genome and also the control of their expressions.

III. The oncogenic and immunogenic potentials of KiMSV(RHHV).

In a long term project we have been investigating the possible oncogenic and immunogenic effects of KiMSV(RHHV) in vivo. Inoculation of 10^2 to 10^3 FFU of KiMSV(RHHV) into newborn Fisher rats did not produce any pathological change among some fifty animals examined at different periods from 14 day p.i. to 15 months p.i. We are currently also examining the presence of specific antibodies in the sera of the inoculated rats after additional inoculations of KiMSV(RHHV). Further studies have also been pursued with thymetomized newborn rats along the same scheme.

In contrast, we have observed that when KiMSV(RHHV) transformed Fisher rat embryonic cells were inoculated into syngeneic newborn or young adult rats, not only was there rapid growth of a capsulated tumor at site of inoculation, but metastases were also seen primarily to the liver. The pathology of such capsulated tumors, as determined from the histological studies of serial sections of the tumor specimens, was minimally differentiated carcinoma.

IV. Virus infection, chromosome abnormalities and neoplastic transformation.

One aspect that has stimulated considerable research interest is the virus-cell interaction that brings about neoplastic transformation. The rat system in this case offers special advantage since its karyotype remains quite stable with the passage of time. Fisher rat embryonic cells (FEC) carry 42 chromosomes. This pattern does not change even with FEC at passage 15. Upon transformation by KiMSV(RHHV) characteristic departures from normal karyotype occur at a all-or-none fashion. The characteristic chromosomal abnormalities, based on results obtained from more than 800 karyotype analyses and over 25 passages of culture, include aneuploidy, breakages, non-disjunctions and translocations, and polysomy. These observations pertaining to the integration of KiMSV(RHHV) genome into the host cell chromosome, tend to support the theory of "Open Replicon" as a basis of viral carcinogenesis.

V. Intracisternal A particle containing neuroblastoma cell lines and their use in studying cell regulation of viral gene induction and expression on the genetic level.

This project is a co-operative effort with Dr. N. A. Wivel, Head of the Ultrastructural Biology Section. In an attempt to analyze the number of copies of stable viral genes in the cell genome and the control mechanism of its expression, we have chosen to work with neuroblastoma. Briefly, three tissue culture clones of neuroblastoma cells derived from the C-1300 tumor in strain A/J mice were found to contain type-C virus specific gs antigen. None of the clones spontaneously released mouse-tropic type-C viruses although one clone, N-4, spontaneously released a xenotropic virus two clones, NB-2A and N-4, could be induced by treatment with 5-iododeoxy-uridine and dexamethasone phosphate to produce B-tropic type-C virus, but clone N-18 failed to respond to various virus induction conditions. Karyotype analysis did not reveal specific chromosome deletions in any of the neuroblastoma clones. The

varying degrees of C-type virus induction and expression among these neuroblastoma clones can best be explained on the basis of evolutionary divergence in regulatory gene control of the neuroblastoma clones in their response to virus induction and expression.

VI. Physicochemical properties of the endogenous DNA transcript of intracisternal A particle reverse transcriptase.

In an on-going project with Dr. N. Wivel the endogenous DNA product of IAP reverse transcriptase was analyzed in detail by various physicochemical methods. Nascent DNA transcript of IAP reverse transcriptase appeared to be covalently linked to an RNA primer. Fidelity of transcription is evident since the DNA transcript hybridized specifically back to the 35-70S RNA of IAP but not with heterologous RNAs. The DNA transcript has an approximated M.W. of 145,000, estimating 350 polynucleotides and base ratios with an excess of dGMP.

Relationship of Research to National Cancer Plan:

Project Area - 10

Approach Elements - 2

Approach - 4

Publications:

Yang, S. S. and Wivel, N. A.: Physicochemical analysis of deoxyribonucleic acid product of murine intracisternal A particle RNA-dependent DNA polymerase. Biochem. et Biophys. Acta, 447: 167-174, 1976.

Wivel, N. A. and Yang, S. S.: Murine neuroblastoma clones with varying degrees of C-type virus expression. Int. J. Cancer 18: 236-243, 1976.

Yang, S. S., Malech, H. M., Wu, R. S. and Woronow, D.: Properties of a transforming virus, KiMSV(RHHV) isolated from a co-culture of rat HTC-H1 cells with K-NRK cells. J. Gen. Virol. (in press) 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05550-09 LCBGY

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Role of Retroviruses in Immunogenesis and Oncogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	T. Log	Sr. Staff Fellow	LCBGY	NCI
	M. J. Rogers	Sr. Staff Fellow	LCBGY	NCI
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COOPERATING UNITS (if any)

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Viral Oncogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.00	1.50

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of the present project is to investigate the role of retroviruses in the etiology of reticulum cell as well as other lymphoid cell neoplasia, and to study the functional markers and surface antigens of tumor cells.

The topics of current interest are: 1) Isolation and characterization of ecotropic and xenotropic retroviruses from SJL/J strain of mouse which exhibit a high incidence of spontaneous reticulum cell neoplasm (RCN); 2) Transplantable RCN exhibiting a killer activity against some other tumors; 3) Distinction between viral structural proteins (VSP) and tumor specific transplantation antigens (TSTA); and 4) Virological and immunological aspects of a nonproducer hemangioma originally induced by Moloney-MSV.

PHS-6040
(Rev. 10-76)

Project Description:

I. An ecotropic virus designated D1-MuLV has been isolated and characterized as reported previously. Since the etiologic role played by this virus in RCN is not yet clear, another endogenous virus which is xenotropic in its tropism was looked for in the SJL/J strain of mouse. After many trials, we finally succeeded in isolating a xenotropic virus from the normal mouse embryo cultures and RCN cultures of SJL/J mouse by co-cultivation with Kirsten MSV-transformed nonproducer human osteosarcoma cell (KHOS) and subsequent propagation in a line of rabbit cornea cells (SIRC). The virus was compared with other known xenotropic viruses such as those from NZB mouse embryos, and AT124 virus that was isolated from NIH Swiss mouse. They were found to be closely related by cross interference tests and neutralization tests, although minor differences may exist. Further work is in progress to characterize this virus and investigate its role in oncogenesis and immunologic reactivities of RCN.

In collaboration with Dr. Aulakh, the D1-MuLV RNA has been extracted, and a complementary DNA (cDNA) was prepared. This will serve as a probe for determination of the degree of homology between the RNA of other murine retroviruses such as Rauscher, Moloney, and Gross, or those from other animal species. This probe will also be used to investigate the presence of D1-MuLV genome in various tumor as well as normal cells. This work is in progress.

II. In the course of investigations on the immunological response of host animals against RCN and lymphocytic leukemias which developed in SJL/J mice neonatally inoculated with D1-MuLV, it was discovered that the lymphoid cell suspension prepared from transplantable RCN showed a marked cytotoxic effect against some other tumor cells in culture. This "killer" effect was not indiscriminatory, but rather selective, and did not require the presence of macrophages. The susceptible target cells included RBL-3, EL4G$^-$, RBL-5, MBL-2, FBL-3 (all of C57BL/6 origin), LPC-1 (BALB/c) and C1.18.4 (C3H). The non-susceptible target cells were D2 (SJL/J), HFL/d-tc (BALB/c), L4946 (AKR), and E^{0}G2 (C57BL/6). Only viable cells of well established D4, RC55, and RC519 of SJL/J RCN were able to show this killer activity. Primary spontaneous RCN or lymphocytic leukemia of SJL/J origin did not show such a killer activity. The killer cell was found to be Fc positive, Thy 1.2 negative, and not affected by pretreatment with anti-Thy 1.2 and complement. Further work is in progress to determine the surface markers on target cells that are recognized by these killer cells. The possibility that the killer activity is exerted by the host cells reacting against RCN is unlikely because SJL/J or C57BL/6 mice immunized with D4 cells did not show cytotoxic activity against RBL-3 or EL4G$^-$ target cells. These data suggest that the transplantable RCN cells were derived from a naturally present, non-T-cell clone that has a cytotoxic effector activity against cells with certain membrane markers. It is postulated that killer cell clones arising spontaneously and destined for immunological surveillance against some (presumably type-C viral, or tumor specific) antigens present on transformed cells have themselves transformed and become a neoplasm, such as RCN. The fact that, in contrast to the RCN of SJL/J mice, the RCN of BALB/c origin did not show this property is interesting in view of the

unique immunological status of SJL/J strain which tends to develop paraproteinemia, dysfunction of humoral antibody production, and impairment of cellular immunity.

The availability of such a natural killer cell clone in the form of a tumor provides a great advantage for studying the properties of natural killer cells and mechanisms of killing processes. The confusion that exists today on the surface markers of natural killer cells may be clarified by analyses of available clones of killer RCN. The fact that, so far, only the established, transplantable line of RCN but not the primary RCN exhibit this killer property is intriguing. This may imply the presence of a selective process in vivo of a clone of killer cell which survives better in the face of inimical forces. The possibility that such a killer cell tumor may exist in human situation is also worth exploring. Further work is in progress to define the membrane characteristics, mode of killing, host control, and immunogenetic processes in development of RCN tumors.

III. In the murine system, efforts to distinguish between VSP and TSTA are still continuing. Anti-VSP (p30, and gp69/71) sera as well as syngeneic anti-crude membrane (CM) sera were used against RBL-5 (virus-producer) and RBL-3 (virus-nonproducer) as target cells in cytotoxicity and membrane immunofluorescence tests. Virus neutralization assay of anti-CM sera and complement fixation test with CM and CS (crude soluble antigen prepared from CM by limited papain digestion) were also performed. It was concluded that p30 expression on RBL-3 membrane was much more marked than that on RBL-5 membrane, although both share a common antigen including gp69/71 and an unidentified antigen which may be TSTA. Preliminary experiments have been carried out to obtain a detergent (Triton X 100)-solubilized antigen from RBL-3 which will inhibit the in vitro cell-mediated cytotoxicity reaction (using ^{125}IUdR-labeled RBL-3 as a target and RBL-3 immune syngeneic mouse spleen cells as an effector). After extraction, the detergent was removed by treatment with SM-2 Biobeads followed by ammonium sulfate fractionation and dialysis. It was found that most marked inhibition of cell-mediated cytotoxicity was obtained with the 30% ammonium sulfate-precipitable fraction. 60μg of this fraction was able to inhibit completely, whereas a purified concentrated suspension of Rauscher-MuLV did not. Further work is in progress to establish the specificity and identity of molecules responsible for this inhibition.

Work has been initiated to develop an enzyme-immunoassay to detect cell membrane antigens with specific antisera. This is essentially an adaptation of enzyme-linked immunoabsorbent assay (ELISA) to retrovirus system. Either the washed target cell suspension in tubes or the solubilized antigen coated on polystyrene Microtiter plates can be used. After reaction with antisera, these are washed and reacted with alkaline-phosphatase-conjugated anti-Ig serum. The enzyme activity remaining on the cell or the antigen-coated plate is measured by the intensity of color reaction after addition of nitrophenyl phosphate as a substrate. We are trying to adapt this test for murine and feline retrovirus systems. Our preliminary data show that ELISA and the complement fixation tests for feline and murine retroviruses have a similar sensitivity. Since ELISA does not require complement, the problem of anti-com-

plementary effects of antigen or antiserum preparations often observed in previous work is eliminated. Compared with membrane immunofluorescence test, ELISA gives a more objective measure of antigen-antibody reaction on the cell membranes. Adaptation of ELISA for detection of FOCMA (feline oncornavirus associated cell membrane antigen) which is currently believed to be an antigen (possibly TSTA) distinct from major VSP gave a satisfactory sensitivity and specificity results. Further work is in progress to adapt ELISA for detection of TSTA in murine system.

IV. A nonproducer cell line cultured from a hemangioma induced by Moloney-MSV in (C57BL/Ka X C3H)F_1 mice has been further investigated. This cell line (HIIHP) was shown to produce no type-C virus and no MSV rescuable by co-cultivation with mouse cells producing helper viruses. However, as published previously, this cell line possesses a tumor specific antigen which can be demonstrated by tumor rejection experiments in which HII tumor growth can be inhibited in M-MSV-immunized syngeneic recipients. Further experiments showed that this transplantation immunity was specific, in that the HIIHP-immunized syngeneic host could not reject a polyoma virus induced tumor, and the polyoma virus-immunized host did not reject HII tumor.

Further experiments were carried out to examine the virological and immunological characteristics of HIIHP cells. If M-MSV or M-MuLV genome is present in HIIHP, it may adversely influence the sensitivity of HIIHP cells to superinfection by M-MSV (M-MuLV) or closely related M-MSV (R-MuLV). This was found to be true in induction of transformation foci after superinfection with these pseudotype viruses. No resistance was observed against superinfection by Harvey-MSV or vesicular stomatitis virus. Furthermore, immunization of (BL/Ka X C3H)F_1 mice with various murine tumor cells such as HFL/d-tc (Friend virus-induced nonproducer tumor), RBL-3, and KiMSV-BALB/3T3 and NIH/3T3 FL gave a significant protection (measured by tumor incidence and/or tumor size) against the challenge inoculation of HII tumor cells. Borderline protection was obtained with FBL-3 and no protection was observed with BALB/3T3, and M-MSV-NIH/3T3FL. These results may be correlated with the amount of expression of some VSP such as sp69/71 or TSTA of FMR specificity. Sequence homology studies by using cDNA of Moloney-RNA as a probe to detect the presence of Moloney-MuLV proviral DNA in HIIHP cell are in progress.

Objective: 3

Approach: 2, 4, 5

Publications:

Chang, K. S. S.: Susceptibility of xenoderma pigmentosum cells to transformation by murine and feline sarcoma viruses. Cancer Res., 36: 3294-3299, 1976.

Law, L. W., Chang, K. S. S. and Ting, R. C.: Immunogenic properties of a nonproducer malignant tumor induced by murine sarcoma virus. Proc. Exp. Biol. Med., 152: 585-588, 1976.

Liu, W.-T., Rogers, M. J., Law, L. W. and Chang, K. S. S.: Properties of RBL-5 leukemia cells cultivated in vitro. J. Nat. Cancer Inst., 58: 1661-1664, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05552-09 LCBGY

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mammalian Cellular Genetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Ching Ho	Visiting Fellow	LCBGY NCI
	G. David Trisler	Post Doctoral Fellow	Armed Forces Radiobiology Research Institute Bethesda

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to conceive, conduct and communicate the results of original research in the area of genetic manipulation of mammalian cells using the techniques of cell fusion, cellular hybridization and cybridization to produce unique material for the molecular genetic analysis of cellular events. Of prime importance in the laboratory now is the genetic system in mammalian mitochondria. The mapping of recombinations between mitochondrial DNA molecules is being studied using restriction enzymes and cRNA-DNA hybridization. The several distinctive subspecies of mouse are proving to be adequately marked material for such studies. Transformation to chloramphenicol resistance of chloramphenicol sensitive cell strains by mitochondrial DNA from chloramphenicol resistant mutant cell strains is being explored as a means to introduce new genes into mammalian cells so that the impact of such genetic surgery can be evaluated.

PHS-6040
(Rev. 10-76)

The activities of the cell genetics unit have been reduced by concentration on a single ambitious project and the loss of key personnel. Two post doctoral fellows left to pursue their careers in new laboratories. One new post doctoral fellow (Visiting Fellow), Dr. Ching Ho, started in mid November 1976 bringing our effort to "full strength". One extramural and two intramural collaborations were discontinued in 1976; these were with Drs. Harry Malech and Nelson Wivel, and Dr. Alan Peterkofsky in Nirenberg's group as well as that with Dr. Igor Dawid at Carnegie Institution in Baltimore.

During this year we have: 1) produced and characterized several valuable chloramphenicol resistant (CAP-r) mutants and fingerprinted their mitochondrial DNA (mt-DNA) - see Table 1; 2) repeated, strengthened and extended our findings (reported last year) that CAP-r mt-DNA could "transform" chloramphenicol sensitive cells (CAP-s) to CAP-r; 3) shown that 3 subspecies of Mus, M. musculus, M. m. molossinus and M. caroli can be used to confer the CAP-r phenotype among one another both by cytoplast fusion and by treatment with CAP-r mt-DNA - see Table 2; the cells generated by these maneuvers are being analysed and should provide unequivocal evidence of the mechanism of mitochondrial transformation; 4) suggested that the mechanism by which nuclear DNA from either CAP-s or CAP-r cells is capable of conversion of CAP-s cells to CAP-r is by way of insertion elements that we hypothesize exist among the repeated sequences of the nuclear DNA (see Table 3) - a preliminary test of this notion as applied to nuclear genes which sought to measure mutation rates for generation of hypoxanthine-guanosine phosphoribosyl transferase (HGPRTase) deficient mutants (i.e., 6-thio guanine resistant) in CHO cells (a collaboration with Dr. Abe Hsie at Oak Ridge) was, however, inconclusive; 5) acquired through the efforts of Dr. Ho two new techniques that will be crucial to our understanding of the new findings made in the past year - they are nick translation of nanogram quantities of mt-DNA using ^{32}P-nucleotides so that small quantities of restriction fragments might be visualized by autoradiography and similarly, labeling with kinase so that the smaller fragments might be seen.

A major part of our effort has been to try to find methods that will allow the production of mutant and "transformed" cells in yields large enough to extract sufficient mt-DNA for analysis, and use in "transformation" experiments. The most efficient methods of cell production use suspension-adapted strains, however, when we transfered one mutant to such a strain the resultant cells, although produced in large quantity, proved to have few mitochondria and correspondingly little mt-DNA. We have fallen back on roller bottle culture methods more suited for culture of large numbers of fibroblasts. The future success of this project will depend on our ability to develop methods for production of large quantities of the mutant cells and of efficient mt-DNA isolation.

The next step in our analysis of mt-DNA, to be undertaken in the coming year, is the use of heteroduplex mapping (electron microscopy of reannealed mt-DNA molecules from mutants and wild type cells) in order to visualize insertions and deletions. We shall start this endeavor as a collaboration but ultimately will have to develop our own EM facility.

Table 1

	Mus musculus DBA, BALB/c MT-OC10, BU-8	*M. m. molossinus* KyK	*Mus caroli* MCH
	estimated molecular weight x 10^6 daltons		
Eco R1	9.5	6.0	4.5
	1.2	2.7	4.1
		1.3	1.2
			0.6
Hind III	9.0	10	6.0
	1.5	0.7	2.1
	1.0		1.7
			0.6

Table 2

	"Transformation" CAP-s → CAP-r Source of mt-DNA: cytoplast/mt-DNA		
	Mus musculus	*M. m. molossinus*	*M. caroli*
Recipient cell			
Mus musculus	+/+	0/0	+/+
M. m. molossinus	+/+	0/0	+/+
M. caroli	+/+	0/0	+/0

Table 3

	Source of purified DNA					
	CAP-r mt-DNA	CAP-s mt-DNA	*E. coli* DNA	$CaPO_4$ O-DNA	CAP-r nucDNA	CAP-s nucDNA
3T3-4EF CAP-s → CAP-r (50µg/ml) "transformation"	+	-	-	-	+	+

Two new extramural collaborations have begun. One involves the use of functional thyroid tumors and secretory cells from normal animals. This collaboration with Dr. Saverio Ambesi-Impiombato and others at the Istituto di Patoligia Generale at Naples seeks to exploit our important new finding that serum free culture media supplemented by hormones permits maintenance of many significant differentiated functions of various kinds of epithelial cells in culture. The second extramural collaboration is with Dr. G. David Trisler, now in Dr. William Shane's laboratory at AFRRI. Trisler helped with the normally segregating neuron x fibroblast hybrids that were developed in our lab over the past 4-5 years. He is taking over the problem with cooperation of two groups at AFRRI since it appears that the work will not be supported at NCI. In addition to corroborating our initial findings of antigens specific to individual neurons (or small groups of them), regionally specific antigens like "theta" have been found to be expressed in a regionally specific manner. The most important application of these neuron hybrid cell lines now appears to be their production of specific brain peptide hormones such as TRF (Thyroid Releasing Factor - demonstrated by two groups in this past year). This new class of hormones has excited much interest of late since they appear to be involved in a wide range of therapeutically significant neural processes from interaction with barbiturate receptors to influences on behavior and mood. The initial discovery of many new members of this new class of hormones may be expected to come from analysis of the synthetic products of these clonal cell lines derived from individual neurons from diverse parts of the nervous system.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05553-09 LCBGY

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structure of Mouse Myeloma Proteins with Antibody Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Rudikoff	Microbiologist	LCBGY	NCI
OTHER:	M. Potter	Head, Immunochemistry Sec.	LCBGY	NCI
	E. Padlan	Visiting Scientist	LMB	A
	D. Davies	Chief	LMB	A
	M. Vrana	Research Assoc.	LCBGY	NCI
	D. N. Rao	Visiting Scientist	LCBGY	NCI
	R. Hooghe	Visiting Scientist	LCBGY	NCI
	B. Mathieson	Staff Fellow	LMI	NIAID
	J. Lunney	Post Doctoral Fellow	I	NCI
	D. Sachs	Head	I	NCI
	J. L. Claflin	Assoc. Prof.	Univ. of Michigan	

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Immunochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objectives of this project are structural determinations of antigen binding myeloma proteins. This analysis includes both three dimensional and primary sequence studies of groups of proteins which bind chemically defined haptenic determinants. These studies will attempt to define the nature of the antibody combinding site for molecules with different binding specificities. The primary sequence analysis of these proteins will provide information as to the pattern of diversity found among closely related proteins which in turn will be important in assessing genetic mechanisms involved in the generation of antibody diversity. In addition, similar studies are being performed on induced antibodies of the same specificity to demonstrate that the myeloma proteins being studied have identical counter parts in the naturally occuring antibody population and are in fact representative of the responses being studied. The identification of these molecules in various mouse strains is evidence for the germ line nature of the particular proteins being studied.

PHS-6040
(Rev. 10-76)

Project Description:

The major project of this laboratory is a continuing study of the primary and three dimensional structure of the antibody molecule. These studies are currently being performed on myeloma proteins produced by plasma cell tumors induced in this laboratory by Dr. Michael Potter. Proteins have been selected for analysis by virtue of their antigen binding specificities and in fact groups of proteins with the same binding specificities have been identified. The present study involves the analysis of three groups of proteins with specificity for phosphorylcholine, levan, and galactan. The comparison of the binding site structure from these proteins will begin to elucidate the types of topographical changes found with differing specificities and to define the molecular basis of antigen-antibody interactions. The comparison of the primary structure of closely related antibodies will permit the evaluation of various proposed mechanisms for the generation of antibody diversity.

1. We have previously reported the complete heavy chain variable region sequences from four BALB/c phosphorylcholine binding myeloma proteins as well as partial light chain sequences. These studies have now been extended to induced anti-phosphorylcholine antibodies in other mouse strains. The light chains from three of the BALB/c myeloma proteins have been shown by isoelectric focusing to be present in seventeen other mouse strains tested. Amino acid sequence analysis of these light chains from A/J mice show conclusively the presence of the same framework and complementarity region sequences as are found in the BALB/c myeloma proteins. The heavy chains from the A/J proteins are also identical to those of the BALB/c myelomas. These results establish the germ line nature of the three light chains being analyzed as well as that of the heavy chain and thus begin to elucidate important aspects of the generation of diversity in this system.

2. A second group of proteins with levan binding activity has been studied to ascertain if the pattern of variation observed among phosphorylcholine binding proteins is applicable to other specificities. Complete heavy chain variable region sequences have been obtained from four of these proteins as well as nearly complete sequences from three of the light chains. In contrast to the phosphorylcholine binding proteins which have light chains from three different subgroups which differ greatly in amino acid sequence the light chains from the anti-levan proteins all belong to the same subgroup and have very few sequence differences between them. The same observation is true for the heavy chains. Whereas both sequence and size differences are found in the complementarity regions of the anti-phosphorylcholine heavy chains the complementarity regions of the anti-levan proteins are all identical. A total of six amino acid differences are found in the frameworks of the four anti-levan heavy chains. Thus, the pattern of variation observed in the anti-phosphorylcholine system cannot be extended as a general pattern to this second specificity.

The group of anti-levan proteins is of additional interest in that members of this group have been shown to possess unique as well as shared or

cross-reacting antigenic determinants (idiotypes). The cross reacting idiotypes have been found to be hapten inhibitable and are presumed to be in or near the combining site. By making recombinant molecules in vitro (exchanging light and heavy chains) between one protein positive for a given idiotype and one negative for that determinant, one of the idiotypes studied in detail has been localized to the light chain of the immunoglobulin. A three dimensional model of this protein has been constructed based on data from our previously reported structure of the M603 protein. By analyzing the sequence differences between the light chains in context of the three dimensional structure we have been able to evaluate the probable molecular basis of this idiotype. These studies are being extended to other idiotypes in an attempt to further define the structural basis of idiotypy.

3. A third group of proteins being analyzed are those with galactan binding specificity. To date nearly complete variable region sequences have been obtained from the light chains of three of these proteins. The variation found is similar to that seen in the anti-levan proteins in that all of the light chains are members of the same subgroup and only very few differences are found between any of the chains. Studies are continuing on the heavy chains from these proteins to permit a comparison with the anti-phosphorylcholine and anti-levan heavy chains. Crystals have been obtained from one of the galactan binding proteins and the three dimensional analysis of this protein is also in progress.

4. Two new projects have recently been initiated which involve studies on cell surface proteins. The first of these problems involves an attempt to isolate and characterize thymocyte antigens. Subpopulations of thymocytes have been found to carry a series of serological markers denoted as Ly 1, 2 and 3. The Ly 1 marker is found on T-helper cells and the Ly 2, 3 markers on cytotoxic and suppressor T cells. These determinants thus are differentiation markers for the various sub-populations of T cells and may be related to the functional characteristics of these populations. A series of T cell tumors have recently been induced in this laboratory by Dr. Michael Potter. These tumors have differentiated Ly phenotypes i.e. tumors that exist, which are Ly 1^-, Ly 2^+ 3^+, Ly 1^+, Ly 2^- 3^-, and Ly 1^+, 2^+ 3^+. We are attempting to use these tumors to internally label the Ly antigens with radioactive amino acids then isolate and characterize the respective markers. Attempts are also being made to raise specific antisera to these markers which can be used to identify and/or eliminate selected sub-populations in various biological experiments.

A second set of cell surface proteins being studied in collaboration with Drs. J. Lunney and D. Sachs are transplantation antigens in inbred miniature swine. Three inbred herds have currently been established and transplantation antigens are being isolated from the respective homozygous herds. The chemical structure of these molecules will be important in understanding the basis of organ rejection and the structural differences between any two of the alleles should provide insight into the molecular basis of this phenomenon.

Publications:

Padlan, E. A., Davies, D. R., Rudikoff, S. and Potter, M.: Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. Immunochemistry. 13: 945-949, 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05596-09 LCBGY

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pathogenesis of plasma cell neoplasia in mice: immunochemical characterization of antigen-binding myeloma proteins; genetic basis of immunoglobulin diversity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Potter	Medical Director (Res.)	LCBGY	NCI
OTHER:	S. Rudikoff	Microbiologist	LCBGY	NCI
	M. Vrana	Res. Assoc.	LCBGY	NCI
	C.P.J. Glaudemans	Chief	LC	NIAMDD
	D. R. Davies	Chief	LMB	NIAMDD
	E. Padlan	Visiting Fellow	LMB	NIAMDD
	H. Morse	Medical Officer	LMI	NIAID
	R. Lieberman	Senior Investigator	LI	NIAID
	J. Minna	Director	HIRBG	NCI
	J. Slack	Biologist	LCBGY	NCI

COOPERATING UNITS (if any)

NIAMDD, NIAID and University of Maryland
Dr. Raymond Dwek, Univ. of Oxford; Dr. Charles Scher, Harvard Univ., Dr. A. Mason Smith, North Carolina Univ. of Greenville

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SECTION

Immunochemistry

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.00	3.00	4.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Mechanisms in mineral oil or pristane induced peritoneal plasmacytomagenesis in the BALB/c mouse are investigated. The role of the granuloma in promoting neoplastic development via adherent cell: tumor cell interaction is being further studied in vitro and in vivo. The possibility of finding a similar dependence in human myeloma will also be explored. A search for the genetic basis of susceptibility continues. The role of type C RNA viruses (e.g. Abelson virus) and endogeneous type C viruses is also pursued.

2. Antigen binding activities of myeloma proteins are determined. Groups of myeloma proteins with similar hapten binding activity are collected, and compared structurally and antigenically with antibodies of similar specificity.

3. The BALB/c mouse has over 28 V-kappa isotypes. Specific antisera are being made to as many of these as possible for purposes of quantitating Ig-gene expression in immune responses and at the B cell level. Genetic markers controlling IgV_H structure and regulation are sought and characterized.

PHS-6040
(Rev. 10-76)

Gene Regulation and Control of Immunoglobulin Structure and Expression

In the last year there has been growing awareness that the allotype (IgC_H) linked immune responses in the mouse could be controlled by a number of different genes acting at different stages in the maturational pathway of the immunoglobulin producing cell. At first many (including ourselves) thought that markers controlling idiotypic differences between two strains could be interpreted to mean a structural gene for a V-region polypeptide had been identified. There are now exceptions, for example, workers in N. Klinman's laboratory (Univ. Pa.) have shown that markers such as the T15 idiotype (originally described in this laboratory) are expressed at the B-lymphocyte precursor cell level but display phenotypic differences in different strains when antibody levels are used as phenotype. It is now apparent that to demonstrate a V-region structural gene marker a number of specific steps will have to be met including the final and important demonstration through amino acid sequence and three dimensional structure analysis of the polypeptides controlling the idiotypes in the different strains. Nonetheless inbred strain differences in level and expression of idiotype can be used as markers to study the entire pathway of immunoglobulin gene differentiation expression. It is envisioned that three levels are technically accessible: 1) the virgin B cell pool which must be obtained from fetal liver or adult bone marrow, this rapidly becomes diluted; 2) functional B cell pool (which contains a disproportion of V_L-V_H clones amplified through an addition of memory, antigen stimulated cells), or cells from the Gut Associated Lymphoid System (GALT); and 3) the plasma cell pool that can be monitored by serum antibodies.

Finally, many types of markers including idiotypes and even sequences can be ascribed to changes that are probably not directly controlled by germ line gene modifications but by "somatic mutations". There is now abundant evidence that both the germ line gene and somatic theories are correct and that both operate at least to create structural diversity though not necessarily as yet proven functional diversity.

This laboratory has been intensively studying 5 groups of antibodies defined usually by their hapten binding specificities: phosphocholine (PC), B1,6 galactan (β6GAL) flagellin (Fla), B2,1 fructosan (Gβ1F) and B2,6 fructosan (Gβ6F). In collaboration with Dr. S. Rudikoff, Dr. M. Vrana, and Dr. N. Rao we have been studying these proteins at the primary structural level: in collaboration with Dr. C. P. J. Glaudemans (NIAMDD) we have been studying the immunochemistry and specificity of hapten binding, and with Dr. E. Padlan and D. Davies (NIAMDD) we have been studying these proteins at the three dimensional levels. Currently it is possible to construct hypothetical three dimensional models of Fv fragments from primary structural data. In cases where crystals can not be obtained, it is possible to confirm inter-atomic distances in the combining site of proposed structures using the technique of nuclear magnetic resonance. Dr. Raymond Dwek of Oxford has pioneered in this method and has confirmed the proposed structure of the MOPC315 binding site. At a recent visit by Dr. Dwek, we established a collaboration with the Oxford group to confirm the proposed structures of J539 a galatan binding myeloma

protein, EPC109 a B,2,1 fructan binding myeloma protein and to begin work on MOPC467 a flagellin binding myeloma protein (the only example of an anti-protein myeloma in our collection. The models of the first two proteins have been built by E. Padlan from the sequences obtained by Drs. Rudikoff, Vrana and Rao. These prototype molecules each have a very distinctive binding site with both marked secondary structural (contour) variations as well as a great difference in the array of amino acid side chains that project into the binding surface. The analysis of these proteins should provide the basis for a more complete understanding of antibody structure and specificity.

We continue to study the antigenic properties, idiotypes of antigen binding myeloma proteins, both from the point of view of finding potential genetic markers but also to gain insight into the problem Ig gene expression outlined above.

One of the continuing efforts of this laboratory has been to systematize the V-kappa isotypes of the BALB/c mouse. Fortuitously the amino acid sequence of the chains up to the first invariant Trp36 provides a means for typing and identifying genes. The mouse has a great diversity of structures (28 V-kappa isotypes so far identified) which are expressed in the myeloma pool and also in antibodies. It has been found by Weigert and McKean that some antisera to V-kappa chains can be isotype specific. This provides a marker for V-kappa isotype expression, and we are actively engaged in preparing idiotype specific sera to all of the V-kappa isotypes. With Mr. John Slack we have identified a Vk-11 isotype specific antiserum that is found on all of the β2,1F binding myeloma proteins, as well as antibodies of the same specificity. This antiserum will be used to study Vk-11 gene expression in serum, and in the 2 above mentioned B cell pools. In collaboration with Dr. S. Rudikoff we shall extend our knowledge on the diversity of V-k-isotypes by partially sequencing, 25 new kappa chains through Trp36. These data hopefully will define the extent of V-kappa isotypy in the BALB/c mouse. The mouse is an ideal species for studying kappa chain diversity because, for evolutionary reasons not understood the framework regions by different isotypes have many sequence differences. This is in contrast to human V_k in only 4 to 5 framework subgroups have been defined. Thus in man, many V_k isotypes have the same amino terminal framework sequence.

Plasmacytomagenesis

We continue to investigate the pathogenesis of peritoneal plasmacytomas induced by the intraperitoneal injections of mineral oil or pristane. The process like other models of neoplastic development appears to have two component processes; first initiation (the process in which the earliest deviation from the normal state begins) and second promotion (those factors that selectively encourage the development of initiated cells to develop to a full blown recognizable tumor).

We continued to study the role of the defective Abelson retrovirus (MLV-A) as an initiator in plasmacytomagenesis. In collaborative studies with Dr. Charles Scher of the Sidney Farber Cancer Center, Boston, Ma., it was found that MLV-A derived from MLV-A induced plasmacytomas was B-tropic (transformed Fv-1^{bb} cells) in contrast to MLV-A derived from lymphosarcomas which is N-B-tropic because of the Moloney Helper virus. We plan this year to determine if B-tropic MLV-A is plasmacytomagenic.

Promotion

We continue to characterize the conditioning (promoting) effects of the mineral-oil granuloma. Induction of a peritoneal oil granuloma is an essential growth condition for transplantation of primary plasmacytomas in BALB/c mice in the peritoneum. This dependence is rapidily lost during continuous transplantation. This year we (M. Potter and M. Cancro) described the requirement for an adherent cell in conditioning. In an in vitro-assay it was also found that the adherence or interaction of plasmacytoma cells with an adherent cell led to increased DNA synthesis. These studies will be extended to determine the kinetics of this interaction in vitro, and to determine if sustained interactions in vitro can lead to the in vitro adaptation of primary plasmacytomas.

Continued study of conditioning in vivo will include studies to determine if T-cells play a role. First, it will be determined if pristane or mineral oil injected nude mice (which lack T-lymphocytes) can, support the growth of primary plasmacytomas. This will be attempted in both BALB/c nu/nu mice and in nu/nu mice of non-BALB/c origin.

We shall also attempt to transplant human myeloma cells, possibly lymphosarcoma cells into conditioned nu/nu mice, to determine if human plasmacytoma cells also have a similar requirement for an adherent cell. The apparent origin of multiple myeloma in the bone marrow cavities strongly suggests a local micro-environment supplies an essential factor for growth. Possibly other tissue components will have to be supplied to nu/nu hosts, and this could include bone marrow stroma. Arrangements have been made with Dr. John Minna, NCI, to obtain cells for these studies. We are currently establishing a nu/nu mouse colony.

Induction of lymphoid tumors by MLV-A in conditioned mice (mice given an injection of pristane 15-45 days before, virus) produces 3 types of tumors: undifferentiated lymphosarcomas (LS) plasmacytic lymphosarcomas (PL) and plasmacytomas. We have established 5 PL tumors in transplant and in collaboration with Dr. E. Premkumar Reddy, Microbiological Associates and Dr. R. M. E. Parkhouse, MRC, Mill Hill have found these tumors. The murine counter part of IgD on their surface. These characterizations of the surface Ig by biosynthetic labelling has thus far been the only indicatory IgD. Newer identification procedures are planned, and also, a means for isolating the idiotype of the Ig on PL tumors will be explored. PL tumors contain a moderate amount of rough ER, yet they do not secrete Ig. The molecular nature of the block in Ig production, that prevents secretion is not under-

A. Initiation

Specific germ line genes, endogeneous and exogeneous viruses and mutagens (carcinogens) are potential initiating factors. In the mouse there appears to be a strong strain-specific component. Only two inbred strains BALB/c and NZB are thus far known to be susceptible to induction. The identity of genes involved in this process are not yet known. Analysis of the Bailey RI strains that were derived from a cross between the resistant C57BL/6 and susceptible BALB/c indicated the two strains with the BALB/c allotype locus were the most susceptible. This year we have completed a study of two BALB/c congenic strains CB-20 and CAL-20 each of which carries a different allotype. Both of these strains appear to be as susceptible, as BALB/c thus the IgC_H locus from two resistant strains is apparently not linked to a resistance gene. The H-2 locus also does not appear to be associated with a resistance gene.

In collaborative experiments with Dr. Carol Cowing NCI we will test this year the susceptibility of backcross progeny in a cross between BALB/c and the resistant DBA/2. These mice will be selected for allotype differences and for Tol-1^a gene(s) that govern resistance to tolerization. This specific trait was chosen for study because both BALB/c and NZB are strains that are relatively difficult to tolerize. BALB/c are Tol-1^{bb}, and DBA/2 are Tol-1^{aa}.

The Tol-1^{aa} gene from DBA/2 was backcrossed for three generations onto BALB/c and second generation backcross progeny segregating for Tol-1^a and Tol-1^b will be tested for plasmacytoma susceptibility.

In further studies looking for the genetic basis of susceptibility other standard inbred strains (RF, STb, BC-8) will be tested. This work has been considerably slowed down in the past 3 years because of problems with laboratory infections that have made it impossible to maintain mice for the 12 to 18 month period following the first injection of oil. Essentially, mice bred in an SPF environment, do not adapt well to dirty mouse rooms, probably because of virulent or potentially virulent bacteria or viruses. We have thus established a production colony of conventional BALB/c, CB-20, CAL-20, mice at Litton Bionetics as a source for four induction experiments. A stable environment has been established at the Litton contract facility and mice have now been maintained for 2 years period, in excellent condition. It is hoped that three to four inbred strains can be evaluated each year.

One question that has arisen in the pristane induction model has been the possibility that a chemical contaminant may be present in the commercially obtained pristane. Dr. Kerry Thomas of the University of Notre Dame prepared for us chemically pure pristane which we have now tested with the commercial preparation which is known to contain impurities. No difference has been noted in the ability of either preparation to induce plasmacytomas.

stood but may constitute a major differentiation step. Understanding the regulation of this step may provide useful information on plasma cell differentiation.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 32555-02 LCBGY

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Induction of Tumor Growth and Immune Response to Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. C. Ting	Medical Officer	LCBGY NCI
OTHER:	L. W. Law	Chief, LCB	LCBGY NCI
	M. J. Rogers	Staff Fellow	LCBGY NCI
	T. Natori	Expert Consultant	LCBGY NCI

COOPERATING UNITS (if any)

Laboratory of Immunodiagnosis, NCI; Laboratory of Cellular Metabolism, NHLI;

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	1.00

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I. Studies of the mechanisms for the induction of in vivo tumor immunity: By employing the ^{125}IUdR release assay and the adoptive transfer experiments, we are able to directly compare the in vitro and in vivo studies, and also to investigate the various phases of immune response in vivo.

II. Humoral regulation of cell-mediated immunity and host control of tumor growth: A humoral immunosuppressive factor can be demonstrated in the tumor bearing hosts in mouse and in man, this factor can suppress the specific cell-mediated tumor immunity. However, host can produce "counter factor" that will neutralize this immunosuppressive factor.

III. Absence of H-2 restriction in T cell-mediated tumor immunity: Strict H-2 homology is not required among the sensitizing cells, target cells and effector cells, to mediated T cell killing. This is true for both the induction phase and the effector phase of the immune response, and in both in vitro and in vivo experiments.

IV. Early detection of antitumor antibodies: A simple, sensitive antibody assay has the potential to be used for early detection of cancer.

Project Description:

Objectives: The objectives of this project are to study: 1) The mechanisms for the induction of in vivo tumor immunity; 2) Humoral regulation of cell-mediated immune response; 3) Host control of tumor growth; (4) H-2 restriction of T cell-mediated tumor immunity; (5) Early detection of antitumor antibody in tumor bearing hosts; (6) Evaluation of isotopic cell-mediated cytotoxic assays; and 7) Immunosuppression by heparin.

Methods Employed: Tumor cells transformed by oncogenic viruses, chemicals or unknown agents are kept in the tissue culture or as transplanted tumors in mice. The isotopic antiglobulin technique (IAT) was used to perform serological studies. The ^{125}IUdR release assay (IRA) was used to measure the cell-mediated cytotoxic responses. The in vivo tumor immunity was tested by immunorejection experiments.

Major Findings: I. Induction of in vivo tumor immunity: the studies of the mechanisms for the induction of in vivo tumor immunity have been hampered by the lack of appropriate methods: on one hand, many of the in vitro assays do not truly reflect in vivo conditions, and on the other hand, it is difficult to separate out different phases of immune response in in vivo experiments for the study. By employing the IRA, we have found a good correlation between the in vitro cytotoxic assay and the in vivo tumor immunity to FBL-3, a Friend leukemia in syngeneic B6 mice. We can further induce the primary and secondary cytotoxic response in vivo by adoptive transfer of lymphocytes. These methods offer us an excellent chance to compare directly the in vitro and in vivo studies and to examine the various phases of immune response in vivo.

II. Humoral regulation of cell-mediated tumor immunity and host control of tumor growth. By employing the FBL-3 tumor system, we have found that ascitic fluids obtained from various murine tumors and pleural effusions from human patients with malignant tumors can promote the growth of FBL-3 in syngeneic hosts, and to suppress the specific cell-mediated tumor immunity. Thus, a immunosuppressive factor(s) is present in the tumor bearing hosts in mouse and in man. However, if the hosts can recover from the immunosuppressive state, they produce a "counter factor" in the sera that can neutralize the effect of immunosuppressive factor. Thus, hosts also appear to play an important role in the control of tumor growth.

III. H-2 restriction of T cell-mediated immunity: It has been demonstrated in both virus-infected mice and in tumor systems that T cell-mediated cytotoxic reactions are H-2 restricted: a strict requirement of compatibility at either the K or D ends of H-2 gene complexes among sensitizing cells, target cells and effector cells. These findings, if they were correct, have important implications on tumor immunology. If such strict requirements of histocompatibility genes exist in all tumors, then it will be impossible to prepare a common vaccine for human tumors or find a common tumor antigen for immunodiagnosis. In working with FBL-3 tumor systems, and a 20 hr IUdR

release assay, we have found that after prolonged incubation time (20 hr or longer), there is no strict requirement of H-2 homology in mediating T cell killing either in the induction phase or effector phase. Furthermore, our work on the inhibition of the T cell killing by sera and soluble antigen also indicate that tumor associated antigens are entirely different from H-2 antigens i.e. only tumor antigen can inhibit cytotoxic reactions, not H-2 antigen, and only antitumor antibodies can suppress the induction of cytotoxic response to tumor associated antigens, not anti H-2 antibodies.

IV. Early detection of antitumor antibodies in tumor bearing hosts: By employing a sensitive isotopic antiglobulin technique, we can detect the antitumor antibodies at very early stages of tumor growth. The changes in antibody levels are also correlated with the host immune status, however, the pattern of changes may vary in different tumors. These findings indicate that a simple sensitive antibody assay can be used for ealy diagnosis of cancer and to be used as a monitoring index to evaluate the host immune status.

V. Evaluation of isotopic cell-mediated cytotoxic assays: We have compared three isotopic assays (^{51}Cr, ^{3}H-proline and ^{125}IUdR) in measuring cell-mediated cytotoxic reactions against tumor associated antigens and alloantigens. We have found that not only the nature of each assay can affect the results, but also the proper choice of baseline, preparation of target cells and methods used for calculation can also have important effect on the interpretation of the results. These findings indicate the need of standardization of the assays that are used in various laboratories.

VI. Immunosuppression by heparin: Heparin has been used as an adjuvant chemotherapeutic agent in cancer patients, the rationale is that heparin can reduce the formation of tumor emboli and thus also reduces metastases. We have found that heparin can interfere with the immune response to tumor associated antigens at both the induction phase and the effector phase. Due to the possible immunosuppressive effect of heparin, its use in cancer patients should be very carefully evaluated.

Significance for Biomedical Research and the Programs of the National Cancer Institute:

The studies on the mechanisms for the induction of *in vivo* tumor immunity have generally been neglected due to various difficulties. Now we have developed techniques which enable us to study the various phases of immune response *in vivo*. Furthermore, the demonstration of the presence of tumor growth promoting factor and immunosuppressive factor in tumor bearing hosts in mouse and in man, and the production of "counter factor" in hosts recovered from immunosuppression indicates that both humoral regulation and host control play important roles in the induction of tumor immunity. The studies of these various problems should help us to understand the mechanisms for the induction of primary tumor in the autothchonous hosts and the host immune response to the tumor. These understandings may help us to find efficient ways to control the tumor growth.

Our findings of the absence of H-2 restriction in T cell-mediated tumor immunity imply that tumor associated antigens are different from H-2 antigens. Thus, it is still possible that a common tumor associated antigen may exists among tumors of the same origin, and this antigen(s) can be used for diagnostic or therapeutic purpose.

If a sensitive technique is properly used, e.g. isotopic antiglobulin technique, one may be able to detect the tumor growth at an early stage and thus successful treatment can be applied on time.

Proposed Course:

I. To study the mechanisms for the induction of in vivo tumor immunity: We shall study the interactions among various population of lymphocytes and between lymphocytes and humoral factors. Emphasis will be placed on the augumentation or suppression of the immune responses by various cellular and humoral factors.

II. To characterize the immunosuppressive factor: Attempts will be made to isolate and purify the immunosuppressive factor that is present in the malignant effusion obtained from both mouse and man. The work on human study will be expanded.

Publications:

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SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBD, NCI
July 1, 1976 through September 30, 1977

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of malignant diseases involving the skin and host's response to these diseases. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 800 patients are seen in consultation each year). The senior staff of the Branch currently includes the Branch Chief, 7 Senior Investigators, 2 Expert Consultants, 1 I.P.A., 5 Clinical Associates, 3 Visiting Fellows and 2 Guest Workers. The Dermatology Branch has made significant contributions to the objectives of the National Cancer Plan. The main research achievements of the Dermatology Branch for the past year are as follows:

Therapy of Skin Cancer and Disorders of Keratinization:

(1) We have initiated exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer and diseases of keratinization.
(2) Sixty-four patients with treatment-resistant disorders of keratinization were treated with oral 13-cis retinoic acid, a synthetic stereoisomer of retinoic acid, for one to 29 weeks. Excellent responses have been observed in patients with congenital ichthyosiform erythroderma, lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris, and cystic acne. However, patients with epidermolytic hyperkeratosis have responded only partially, and patients with psoriasis, nevus comedonicus, X-linked ichthyosis, and Netherton's syndrome have either not responded or have worsened. One patient with the nevoid basal cell carcinoma syndrome improved considerably with marked reduction in size of his skin tumors after therapy with oral 13-cis retinoic acid. Commonly observed side effects included cheilitis, facial dermatitis, xerosis, skin fragility, and conjunctivitis. The mechanism by which this synthetic retinoid alters these disease states is not clear but may be related to the observed ability of vitamin A to affect glycoprotein synthesis and epithelial differentiation.
(3) The profound beneficial effect of 13-cis retinoic acid in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelia, could be successfully treated with the synthetic retinoids. These patients, who have successfully responded to 13-cis retinoic acid, may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

(1) The epidermis offers a unique opportunity for the study of tumors not only because of the quantitative importance of this tissue but also because of its assessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and

biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of the keratin subunits in a well defined cell culture system will facilitate studies of carcinogenesis in vitro. In addition, the finding of large amounts of actin protein in epidermal tumors, and the development of suitable immunoassay procedures for it is of potentially great significance. (2) The seven polypeptide chains which comprise the subunits of the keratin filaments of normal bovine epidermis have been isolated and individually characterized by standard protein chemical techniques. The stoichiometry of recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the X-ray diffraction analyses. The X-ray diffraction data implies the filaments certain region of coited-coil α-helix. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins are being isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The synthesis of the keratin filament subunits by the mouse epidermal cells implies the presence within the cells of the complete protein biosynthetic apparatus, and this system is therefore potentially very useful for studying control of differentiation in the epidermis.

Studies of DNA Repair in Normal Human Cells and in Cells from Xeroderma Pigmentosum Patiants

(1) UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor develop in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an unusually high incidence of cancer such as ataxia telangiectasia will of course further increase our understanding of the relationships between DNA repair processes and carcinogenesis. XP patients' sun-exposed skin ages much more rapidly than normal human's skin. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and these aging phenomena in XP. Thus, our studies of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans.

(2) We have studied post-UV colony forming ability (CFA) in irradiated XP fibroblasts from each of the six genetic forms of XP. A striking correlation between the degree of reduction in post-UV CFA and the presence and severity of XP-associated neurological abnormalities has been found. These results constitute the first strong evidence for a relationship between the effective level of DNA repair and the severity of a hedero-degenerative condition of the nervous system. These results are relevant to the theory of "intrinsic mutagenesis" which hypothesizes that error-proneness in key DNA repair or replication enzymes is responsible for carcinogenesis and for a variety of degenerative diseases as well as for normal aging in various human tissues. We have confirmed recent brief reports that cells from patients with Cockayne's syndrome, a syndrome consisting of cachectic dwarfism, neurological abnormalities and photo-sensitivity, have decreased post-UV CFA. In addition, we have found that Cockayne's syndrome patients have a normal pressure hydrocephalus which is probably the cause of some of their neurological abnormalities. Studies in our lab have confirmed reports of decreased post-X-ray CFA in cells from patients with ataxia telangiectasia, a disease in which patients have an increased rate of cancer, plus degenerative findings in the immune and nervous systems. Such studies are now being performed in parallel with post-UV CFA assays on XP cells, Cockayne cells, and cells from patients with other photosensitive and or degenerative disorders.

Virologic Studies

(1) Warts represent a viral induced tumor of man. A closely related virus, the Shope papilloma virus of rabbits, will induce benign tumors in some rabbits and malignant tumor in others. The squamous cell cancers of patients with epidermodysplasia verruciformis may be induced by wart viruses, as may tumors of other patients. Methods are being developed which should not only permit the screening of tumors for wart viral material, but also facilitate attempts to propagate the virus in tissue culture. Assuming that the analogy with the Shope papilloma virus is correct, propagation of the virus in tissue culture would facilitate the investigation of the molecular mechanisms which result in a benign proliferation of cells in some instances and malignant transformation in others.

(2) Studies to date indicate that viruses from plantar warts have at least 2 different restriction enzyme digestion patterns.

(3) In addition, virologic studies show that an untransformed mink cell which contains the Moloney murine sarcoma viral genome will undergo transformation on treatment with physiologic concentrations of dexamethasone or other glycocorticoids. The extent of transformation correlates with the levels of sarcoma specific viral RNA and with viral p30 protein. Transformation depends on the continued presence of glycocorticoid, since its removal results in loss of transformation and a decrease in viral expression to pretreatment levels.

(4) Finally, it has been found that a sensitive quantitative 10 day transfection assay for murine sarcoma and leukemia viruses have been developed for use with unintegrated and integrated viral DNA. The efficiency of transfection depends on several critical parameters: size of infectious DNA, size of carrier DNA, concentration of DNA, cell density, ph of precipitation medium, and the use of dimethylsulfoxide. With this technique, DNA can be titrated almost as easily as intact virus.

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

(1) Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called autoimmune phenomena. We have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have centered around the mechanisms of tissue destruction, ultrastructure, and ultrastructural localization of antibodies. Gastrointestinal abnormalities, similar to those seen in gluten sensitive enteropathy, occur in dermatitis herpetiformis and are under active study. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of the effect of sulfones on neutrophilic exudative disorders. As sulfones dramatically alleviate dermatitis herpetiformis and erythema elevatum diutinum we are using animal models of inflammation and serum and blister fluids from patients to study their effect on complement activation, deposition and neutrophilic chemotaxis. Alterations in collagen synthesis in inflammatory disease and identification of alloantigens on epidermal cells are other integral parts of the overall program.

(2) The sera of patients with vitiligo associated with multiple endocrinopathies contain antibodies to melanin producing cells.

(3) Langerhans cells which comprise a small population of epidermal cells, bear C3 and Fc receptors as well as alloantigens which are Ia associated.

Studies on Mechanisms of Cellular Immunity in Man

(1) Our combined in vitro and in vivo controlled methods for the study of cell-mediated immunity in man, have allowed us to initiate basic immunopharmacology and immunogenetics approaches for the study of a variety of diseases and has already resulted in several practical diagnostic procedures. Additional studies promise a method for selecting effective immunotherapeutic agents against specific cancers.

(2) In collaboration with the Diabetes Branch we have discovered a new form of diabetes with a defect in the affinity of insulin-receptors. Preliminary evidence suggests that this defect may relate to circulating plasma factors.

(3) A method has been developed (clonal priming) for defining specificity and cross reactivity at the T cell level. The clonal priming reaction offers a practical, ethical, in vitro, quantitative approach for systematic study of relationships recognized as foreign by immune cells. Concepts of cross reactivity, organ- and tissue- specific antigens, autoimmunity, tolerance and immunohomeostasis have been well developed. However, progress has been hampered by lack of an experimental approach to directly compare tissue, differentiation, tumour, microbial (including viral) and other environmental antigens at the level of cellular immunity. With clonal priming, it may now be possible to pinpoint agents implicated in a variety of inflammatory and neoplastic diseases in which cellular immune discrimination plays a role.

Biochemical Characterization of Mammalian Melanosomes

(1) The abnormal proteins produced in malignant melanoma are being studied: these studies are aimed at elucidating the mechanism of formation of these

atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential.
(2) In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, gly, ala, leu).
(3) The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. Melanomas are usual among cancers since they do not result in complete differentiation of the affected melanocyte, but allow this cell's specialization, i.e. melanogenesis, to continue. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal. There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanosomes. An analogous situation of altered proteins has been described in several other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. The similarity of these proteins suggests that the unique proteins present in the malignant melanocyte are an aberrant form of the analogous protein in the normal melanocyte, although the mechanism effecting this has not yet been elucidated.

Applications of SEM to Soft Biological Tissues

(1) SEM reveals fine surface structure with unparalleled three-dimensionality, depth of field, and sampling capability. Combined autoradiographic studies for the first time demonstrate that interphase stages in the cell cycle (skewed by neoplastic growth) may have little or no influence on the topography of cells in monolayer culture.
(2) The widely held notion that the topography of cells in monolayer culture changes predictibly as they progress through interphase stages of the cell cycle has been based entirely on studies of synchronized cell populations that have not been controlled for the impact of forcible synchronization procedures on cell form. Cinephotomicrography reveals that under the conditions ^{3}H-TdR addition, fixation procedures, and processing for SEM do not appreciably alter CHO cell topography. Thus, the surface morphology of cells in S phase (identified by autoradiographic assay of ^{3}H-TdR incorporation into cells that have been scanned) can be distinguished from cells not in S phase without perturbing their growth. Our initial results clearly indicate that the S phase of the cell cycle has little or no influence on the topography of these cells in standard asynchronous monolayer culture.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3630-07 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Morphologic and Biochemical Effects of Vitamin A and Analogs on Chick and Human Skin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	P.V. Bhat	Visiting Fellow	Derm NCI
	P.M. Steinert	Expert Consultant, Biochemist	Derm NCI
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COOPERATING UNITS (if any)
Experimental Pathology Branch

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.68	0.68	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project proposed to morphologically and biochemically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in embryonic skin, normal skin, and in benign and malignant lesions of skin.

Chick metatarsal skin, when cultured in vitro, is stimulated to undergo mucous metaplasia by vitamin A acid. Ultrastructural analysis alone cannot determine whether a post-translational effect, a genomic effect, or an effect of vitamin A acid on the mesenchyme is the primary mechanism involved in the inhibition of keratinization and induction of epidermal mucin biosynthesis by vitamin A acid.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanism by which vitamin A alters epidermal cell differentiation with resultant inhibition of keratinization in embryonic skin, normal adult skin, and in benign and malignant lesions.

3) To study the early changes of vitamin A induced mucous metaplasia at the ultrastructural level. In particular, to determine if epidermal cells are capable of both mucin and keratin production, or whether the two processes are mutually exclusive.

4) To define certain dermatologic disorders of keratinization at the biochemical level.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) Metatarsal shank skin (devoid of feathers) is obtained from 13-14 day chick embryo.

2) 0.2-0.4 mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier's Disease (NCI-3643) has been used to date.

3) 3-4 mm punch biopsies of normal and diseased human skin (NCI-3643).

Methods Employed:

1) Chick metatarsal skin is cultured in vitro and stimulated to undergo mucous metaplasia by vitamin A acid. Specimens are evaluated for mucin production by the use of special stains, such as PAS (with and without diastase), Alcian Blue, Toluidine Blue, and Colloidal iron. Specimens are also examined under the transmission and scanning electron microscopes. In addition, the scanning electron microscope is being used to study in ovo skin scale and feather development, and their inhibition to vitamin A acid in vitro. The effect of retinyl acetate on the morphology of mouse epidermal cells in culture is also being studied.

2) Chick embryo skin or skin from patients with Darier's Disease was incubated both with and without Vitamin A in a water bath shaker in the presence of labelled precursors. After incubation, specimens were prepared for biochemical extraction and analysis.

Major Findings:

1) The changes observed in vitamin A acid treated cultures are both dose-and time-dependent and are reversible when explants are transferred to control medium. Using 20 i.u. (6.7 ugm) vitamin A acid per milliliter of culture media, it was found that maximum mucous metaplasia occurred with 3 days of in vitro incubation. As little as 1 I.U. produced metaplasia in 6 days. Metaplasia was inhibited or reduced greatly at room temperature, or in the absence of serum.

2) Microscopically, the control specimen keratinized normally with the formation of thick bundles of tonofilaments, keratohyalin granules and a multi-layered stratum corneum. Vitamin A acid treated specimens did not undergo keratinization. The periderm sloughed prematurely. There was no tendency of the columnar basal cells to horizontally flatten as they approached the surface. Neither stratum corneum nor keratohyalin granules were formed. Tonofilaments were present in small, fine bundles. In addition, there was increased folding of the nuclear membrane, and increased amounts of Golgi elements, rough endoplasmic reticulum and polyribosomes. Mucin was present in the cytoplasm as membrane-bound electron dense material. Other transmission E.M. findings included desmosomal cleavage, formation of tight junctions, and basement membrane gaps with elongated processes of epidermal cells extending into the dermis between the gaps. Direct contact between fibroblasts and collagen fibers, with the epidermal cell processes is seen. The germinative cells of the epidermis are small, darkly staining, and increased in number. The more superficial spinous cells are large and lightly staining suggesting a distinct and direct effect on germinative cells.

3) In addition to mucin formation there is a glandular metaplasia in the epidermis with formation of acini with intercellular canaliculi characterized by tight junctions, brush borders, and dense contents. This morphologic change has not been demonstrated previously.

4) Vitamin A induced mucous glandular metaplasia was inhibited by the addition of puromycin or cycloheximide to the culture medium; actinomycin D, hydroxyurea, and colchicine failed to inhibit this process, thus suggesting that protein synthesis may be necessary for metaplasia, while mitosis, and DNA and RNA synthesis may not.

5) Scanning electron microscopy revealed that metaplastic changes were visible as early as one day after vitamin A acid exposure. Individual epidermal cells lost attachments to their neighbors, became spherical,

and developed surface villi. Selective basal cell layer effect was observed in 17-day chick embryos treated with vitamin A. Although the superficial stratified squamous layer of the epidermis was unresponsive to vitamin A, it was thrown into large folds by the hyperplastic masses of underlying metaplastic cells.

6) Chick skin scale formation is inhibited by vitamin A acid.

7) We have studied the effects of vitamin A on embryonic chick skin at the biochemical level. DNA synthesis as measured by H-Thymidine incorporation declines progressively and becomes negligible by the 6th day after explanation. In contrast to other systems, vitamin A does not stimulate DNA synthesis. On the other hand, overall rate of protein and RNA synthesis is not affected by placing skin in culture. Electrophoretic protein analysis indicates that synthesis of several major polypeptides is turned off by vitamin A administration, but to date, we have been unable to find the stimulation of any protein that might represent the prosthetic group of mucin (a glycoprotein). On the other hand, when H^3-fucose (one of the sugars in epithelial mucins) is used as a precursor, it can be seen, again with the aid of gel electrophoresis, that several glycoproteins are decreased in synthesis with vitamin A administration, while one low molecular weight glycoprotein (which is absent in normal tissue) is synthesized very actively S-sulfate incorporation into macromolecules of the epidermis, presumably sulfated mucopolysaccharides, is also stimulated by vitamins. While further work is needed, these results would seen to indicate that vitamin A causes mucus metaplasia primarily by inhibiting keratinization, and at the same time allowing mucin formation to occur, presumably by the glycosylation of pre-existing protein in the chick epidermal cell.

8) Preliminary electrophoretic analyses have indicated that several major protein components found in normal skin are lacking in involved skin in Darier's. This data, might indicate that either certain metabolic processes are inhibited in patients or that excessive degradation of the protein components has occurred. Further work is being done to resolve this issue in Darier's disease and other cutaneous disorders of keratinization.

9) Initial efforts have been made to develop a microsomal membrane system from the epidermis which will use labelled precursors, such as mannose and galactose, for the biosynthesis of mannosylretinylphosphate and galactosylretinylphosphate, which may be the initial intermediates in the formation of glycolipids and glycoproteins. Once this microsomal membrane system is standardized, it will be used to study the glycosyltransferase activities in normal human skin, basal cell carcinoma, and cutaneous disorders of keratinization. The effects of oral administration of the retinoids on the glycosyltransferase activity of these tissues will also be studied.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A-induced redifferentiation, both in human and chick skin, may serve as an excellent model for investigations of cellular control mechanisms which relate to carcinogenesis. The fact that carcinogen metabolism and carcinogenesis are influenced very markedly by vitamin A deficiency directly links research on vitamin A epithelial effects to cancer research.

2) Furthermore vitamin A is of value in the treatment of malignancy. The mechanisms of action of vitamin A in inhibiting keratinization may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of the above material.

2) The effect of vitamin A and its derivatives on the glycosyltransferase activities of normal and diseased skin will be studied.

3) Further attempts will be made to characterize the epidermal proteins in cutaneous disorders of keratinization.

Publications:

1) Peck, G.L.: Differentiation and metaplastic properties of stratified squamous epithelium in cholesteatoma - First International Conference. (eds. B.E. McCabe, J. Sade, and M. Abramson) Aesculapius Publishing Company, Birmingham, Alabama, 1977.

2) Peck, G.L., Elias, P.M. and Wetzel, B.: Influence of Vitamin A on differentiating epithelial in the Biochemistry of Cutaneous Epidermal Differentiation (eds. M. Seiji and I.A. Bernstein). University of Tokyo Press, Tokyo, Japan, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3632-07 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cutaneous Manifestations of Graft-Versus-Host Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	T.J. Lawley	Clinical Associate	Derm NCI
	H.M. Moutsoupoulos	Visiting Fellow	NIAMDD
	A.B. Deisseroth	Section Head	PO NCI

COOPERATING UNITS (if any)
Experimental Hematology Section, Pediatric Oncology Branch, NCI
Laboratory of Microbiology & Immunology, NIDR

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.01	.01	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing clinical study of patients with acute and chronic Graft-Versus-Host Disease (GVHD) has led to the discovery of one patient with acute GVHD whose cutaneous eruption progressed to Toxic Epidermal Necrolysis and another patient with chronic GVHD who developed cutaneous scleroderma and a sjogren's like syndrome. Histological and ultrastructural studies are being done on these patients and on other current patients with GVHD.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the pathogenesis of the cutaneous manifestations of Graft-Versus-Host Disease (GVHD) particularly Toxic Epidermal Necrolysis (TEN), scleroderma and sjogren's syndrome and to investigate the relationship between them.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone and in combinations) in the cure and palliation of cancer.

Methods Employed:

1) Examination of patients with aplastic anemia or acute lymphocytic leukemia, treated with allogeneic bone marrow transplantation, who developed GVH with skin manifestations.

2) Examination with the electron microscope of multiple skin biopsies taken at various time intervals and at various skin sites.

Major Findings:

The cutaneous manifestations of acute GVHD are initially morbilliform and, subsequently, scarlatiniform.

1) Clinical Findings: One case was observed in which the initial morbilliform eruption of GVHD progressed to sheet-like desquamation of the epidermis, typical of TEN. Another patient with chronic GVHD developed sclerodermatous skin and xerophthalmia and xerostomia resembling sjogren's syndrome.

2) Electron microscopic findings: The initial pathologic events in GVHD in the patient who developed TEN in the basal layer of the epidermis, and it appears that the mononuclear infiltrate may play a significant pathogenetic role. Mitochondrial swelling, coarsening of tonofibrils and increased prominence of ribosomes were noted in all basal cells. The nucleu appeared contracted, with more convoluted nuclear membranes; increased density of chromatin was noted. Lymphocytes were occasionally seen within the basal layer, but only rarely above. Increased numbers of Odland bodies and abnormal keratohyalin granules were noted in the superficial epidermis.

Significance to Cancer Research:

1) Immunosuppressed patients, including treated cancer patients, who are transfused or transplanted with immunocompetent cells, may expect

to develop GVHD. In view of our experience, we may now expect some of them to develop TEN, cutaneous sclerosis, or a Sjogren's-like syndrome as well.

Proposed Course of Project:

Electron microscopic examination is continuing. Specimens from new patients with skin manifestations of GVHD are also being studied.

Publications:

1) Lawley, T.J., Peck, G.L., Moutsopoulos, H.M., Gratwohl, A.A., Deisseroth, A: Scleroderma and a sjogren's like syndrome in a patient with chronic graft-vs-host disease. Ann. Int. Medicine (submitted).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3638-08 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies of DNA Repair in Normal Human Cells and in Cells from Xeroderma Pigmentosum Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Robbins	Senior Investigator	Derm NCI
OTHER:	A.D. Andrews	Clinical Associate	Derm NCI
	L.B. Lyons	Expert Consultant	Derm NCI
	F.W. Yoder	Clinical Associate	Derm NCI
	R.A. Brumback	Clinical Associate	MN NINCDS
	W.C. Cheng	Visiting Fellow	MB NCI
	J. Whang-Peng	Medical Director	MB NCI
	R.S. Day III	Research Physical Scientist	CH NCI
	K.I. Snowden	Gnotobiotics Unit Head	VRB DRS
	S. Potkay	Chief, Small Animal Section	VRB DRS

COOPERATING UNITS (if any)
Medical Neurology Branch, NINCDS; Medicine Branch, Clinical Oncology Program, DCT, NCI: Chemistry Branch, DCCP, NCI: Small Animal Section, VRB, DRS.

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.5	3.8	3.7

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases, carcinogenesis, and ageing. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP), all of whom have defective DNA repair plus the clinical abnormalities of premature ageing of the skin and cutaneous malignancies. We measure the DNA excision repair process by assays of UV-induced unscheduled DNA synthesis and are using these assays to study the UV dose response of the excision system and the effect of in vitro aging on this system. We assess the biological effectiveness of DNA repair with assays of post-irradiation colony-forming ability and have found a striking correlation between the level of effectiveness of DNA repair in XP and the severity of XP-associated neurological abnormalities. We are studying the relationship of UV-induced sister chromatid exchanges to the repair defects of XP. We are extending our work to include cells from patients with other diseases in which DNA repair defects are claimed or suspected, such as Cockayne's syndrome, ataxia telangiectasia, Werner's syndrome, Rothmund Thompson syndrome and progeria. We have established lymphocyte cell lines (long-term lymphoblast lines) from all six genetic forms of XP. These lines are useful in radiation-sensitivity and biochemical tests.

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the six genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected; to determine the relationships between the function of such DNA repair processes and the clinical findings of photosensitivity, premature aging, and skin or other cancers.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Most cells are now obtained from established cell repositories. They are also obtained from hospitalized patients and outpatients at the NIH and occasionally as gifts from other investigators. Cells currently under study include dermal fibroblasts, peripheral lymphocytes, and lymphocyte cell lines (long-term lymphoblast lines).

Methods Employed:

Rates of excisional DNA repair are determined by using scintillation spectroscopy and autoradiography to measure the unscheduled incorporation of tritiated thymidine into X-ray or ultraviolet (UV)-irradiated cells. The biological effectiveness of repair is tested by assays of post-irradiation colony-forming ability (CFA) and by post-irradiation trypan blue viability tests. Sister chromatid exchange, before and after irradiation, is determined by Hoechst staining and examination of cells in the appropriate metaphase; the fluorescence-plus-giemsa technique may be utilized in the future. Cells are cloned, and the cloned cells are tested in post-irradiation colony-forming tests to determine the origins and bases for different components of such curves. Cockayne's syndrome patients have been evaluated by appropriate standard diagnostic tests in an attempt to define and to determine the bases of the disease's clinical manifestations particularly with the aim of being able to provide information leading to amelioration and/or cessation of certain of their abnormalities. Photobiological tests in vitro, similar to those used with XP cells, are being conducted on cultured cells from patients with Cockayne's syndrome. XP and normal human cells are being irradiated and injected into nude mice to determine if radiation-induced malignant tumors will develop. Our very sensitive autoradiographic techniques for determining the uptake of tritiated thymidine into DNA is being employed to detect mycoplasma contamination of human cells in tissue culture.

Major Findings:

1) We have studied post-UV colony-forming ability (CFA) in irradiated XP fibroblasts from each of the six genetic forms of XP. A striking correlation between the degree of reduction in post-UV CFA and the presence and severity of XP-associated neurological abnormalities has been found. These results constitute the first strong evidence for a relationship between the effective level of DNA repair and the severity of a heredo-degenerative condition of the nervous system. These results are relevant to the theory of "intrinsic mutagenesis" which hypothesizes that error-proneness in key DNA repair or replication enzymes is responsible for carcinogenesis and for a variety of degenerative diseases as well as for normal aging in various human tissues.

2) The post-UV CFA studies of certain XP strains have revealed a somewhat greater UV resistance at high UV doses than at low UV doses. Cloning experiments are in progress to determine if such results are due to the presence of two distinct cell populations or are a reflection of dose-dependent differences in repair efficiency intrinsic to all the cells in these strains.

3) We have confirmed recent brief reports that cells from patients with Cockayne's syndrome, a syndrome consisting of cachectic dwarfism, neurological abnormalities and photosensitivity, have decreased post-UV CFA. We have studied cells from six unrelated patients and are establishing additional new strains. The studies have already revealed that there are significant differences among Cockayne strains in the degree of reduction in post-UV CFA. No defects in UV-induced unscheduled DNA synthesis have been found, however, in any of these strains.

4) A clinical study of patients with Cockayne's syndrome has been completed. Complete work-ups of all abnormal clinical findings in such studies have given us an important opportunity to relate the clinical manifestations of this syndrome to the laboratory findings, as has been, and is being, done with XP patients. We have found that Cockayne's syndrome patients have a normal pressure hydrocephalus which is probably the cause of some of their neurological abnormalities.

5) Studies in our lab have confirmed reports of decreased post-X-ray CFA in cells from patients with ataxia telangiectasia, a disease in which patients have an increased rate of cancer, plus degenerative findings in the immune and nervous systems. Such studies are now being performed in parallel with post-UV CFA assays on XP cells, Cockayne cells, and cells from patients with other photosensitive and or degenerative disorders.

6) We have succeeded in establishing a total of 25 lymphocyte cell lines (long-term lymphoblast lines) from the six genetic forms of XP. Work to date with several such lines indicates that they retain the same level of diminished DNA repair as the fresh peripheral lymphocytes from which they

arose and that they have decreased post-UV survival. Further characterizations of all such lines is underway. These lines should be valuable in attempts to characterize and purify the defective DNA repair enzymes in XP. Such lines have also been established from patients with Cockayne's syndrome and ataxia telangiectasia.

7) In collaboration with Dr. Weng-Chow Cheng and Dr. Jacqueline Whang-Peng of the NCI, studies of UV-induced sister-chromatid exchanges are being performed using normal, XP and Cockayne's syndrome lymphocyte cell lines. It is already clear that UV-induced sister chromatid exchanges are greater in most XP lymphocyte cell lines than in normal lines.

8) Studies in co-operation with Dr. Rufus Day, III, of the NCI are being conducted on the Cockayne cells to determine if these cells have reduced repair of UV-irradiated adenovirus 2.

9) Attempts are being made to accomplish in vitro malignant transformation of XP and normal fibroblasts by UV light. The irradiated cells are injected into nude mice to determine if malignant tumors will form. If successful, the results of such work, when correlated with our knowledge of DNA repair efficiency and UV mutability, should yield important new information concerning the interrelationships of DNA repair, mutagenesis and carcinogenesis.

10) Autoradiographic studies of XP cells after UV irradiation have shown that a very small (ca 1% of normal) but detectable amount of UV-induced unscheduled DNA synthesis occurs in cells from patients in complementation group A, the genetic form of XP having the least such synthesis. This work has demonstrated the great sensitivity of our assay system; no other laboratory has been able to measure such small amounts of unscheduled DNA synthesis.

11) Autoradiographic screening of cells for the presence of mycoplasma (detected as 'cytoplasmic' incorporation of tritiated thymidine) has been shown to be of great value, especially when dealing with those species of mycoplasma not easily detected by standard culturing techniques. Such screening is of great importance to all work on DNA repair as mycoplasma contamination can significantly alter the results of repair assays and, if undetected, could lead to erroneous conclusions. The sensitivity of our detection method is being compared to standard fluorescent and culture techniques for detecting mycoplasma.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells

have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor develop in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an unusually high incidence of cancer such as ataxia telangiectasia will of course further increase our understanding of the relationships between DNA repair processes and carcinogenesis.

XP patients' sun-exposed skin ages much more rapidly than normal human's skin. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes and these aging phenomena in XP. Thus, our studies of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

1) Andrews, A.D., Barrett, S.F. and Robbins, J.H.: Relation of DNA repair processes to pathological aging of the nervous system in xeroderma pigmentosum. Lancet I:1318-1320, 1976.

2) Kraemer, K.H., Andrews, A.D., Barrett, S.F., and Robbins, J.H.: Colony-forming ability of ultraviolet irradiated xeroderma pigmentosum fibroblasts from different DNA repair complementation groups. Biochimica et Biophysica Acta 442:147-153, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3643-07 D

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Surgical Treatment of Darier's Disease (Keratosis Follicularis)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	P.B. Chretien	Surgery Branch	NCI

COOPERATING UNITS (if any)

Surgery Branch, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.10	.10	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with severe, recalcitrant, confluent Darier's disease were treated with either dermabrasion, dermatome stripping, free-hand knife techniques, or electrocautery. Results indicate that surgical treatment can produce long lasting (over 5 years to date) disease free remissions. Furthermore, wound healing, especially epidermal migration, is more rapid in the diseased skin than in uninvolved skin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To observe the response of Darier's Disease to surgical therapy.

2) To study the effect of experimental wounding of both involved and uninvolved skin.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study of the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

Normal and diseased skin in patients with Darier's Disease.

Methods Employed:

1) Dermatome stripping in patient 1. Strips of skin were taken from a 62-year-old female patient with Darier's disease using a Castroviejo opthalmologic keratotome (Storz Instrument Co.). Strips were approximately 2 cm. in width, 5 cm. in length and 0.2 mm. thick, determined by using a 0.2 mm. spacer in the shaving head. The wounds were then left open, measured and photographed daily.

Incisional wound healing. Multiple linear incisions, approximately one cm. in length, were made in diseased skin areas of the right anterior forearm and in clinically uninvolved skin of the left anterior forearm. Four mm. punch biopsies were taken at zero time, and 3, 6, 12, 18, 24, 48, and 72 hours after wounding.

2) Dermabrasion therapy in patient 2. A 26-year-old man with hypertrophic cornifying Darier's disease specially severe on arms and legs was treated with surgical dermabrasion under general anesthesia. Rate of wound healing was measured by serial 6 mm punch biopsies of circular defects produced by 3 mm punches, and by serial punch biopsies taken from dermatome stripped sites of diseased and uninvolved skin punches.

3) Dermatome stripping in patients 3 to 5. Two women and one man with severe, confluent, inflammatory, vesicular Darier's disease were treated with dermatome stripping of legs or buttocks under general anesthesia. Rate of wound healing was measured by the methods used in patient 2. One of these women also had dermabrasion of the face and excision of severely diseased labia majora.

Major Findings:

1) Dermatome stripping in patient 1. Involved sites healed more rapidly than uninvolved skin following dermatome stripping. Two weeks after stripping, the uninvolved site remained larger and more erythematous than the involved site. Furthermore, a zone approximately 10 cm. wide of previously diseased skin around stripping sites on the left hip was observed to have clinically regressed by this time. Clinical regression of involved skin surrounding dermatome stripped sites was repeatedly observed.

Five months after the initial stripping experiment, the patient had a generalized exacerbation of her disease, which spared previously dermatomed sites of uninvolved skin. Histologically, the epidermis and pilosebaceous apparatus appear normal except for diminution of the rete ridge pattern and flattening of the papillary dermis.

Incisional Wound Healing. Superficial linear incisions healed more quickly in diseased sites (12 hours) than in uninvolved sites (48 hours) indicating accelerated epidermal migration.

2) Diseased sites healed more rapidly than uninvolved sites, using both methods measuring rate of wound healing. 3mm defects in diseased skin healed in 2 d, and in uninvolved skin in 3 d. The patient responded well to dermabrasion and had less than 10% recurrence after 11 months of observation. Peripheral clearing, as seen in patient 1, was not observed.

3) Surgical treatment was well tolerated in all patients producing long lasting (over 5 years) disease free remissions. Peripheral clearing has occurred in 3 of 5 patients following dermatome stripping.

Surgical treatment is successful in relieving the symptomatic manifestations of Darier's disease most probably because the pathophysiology of the disease may be related to an altered dermoepidermal interaction. Since surgery removes papillary dermis as a "margin" to insure complete excision of the elongated epidermal ridges, and since the few areas of recurrence of persistent disease occurred where excision was not sufficiently deep into the dermis, elimination of dermoepidermal interaction, or more particularly, the capillaries in the papillary dermis may be required for elimination of the disease. Histologically, the regenerated skin in our patients has an absent or markedly reduced papillary dermis. This lends further support to the suggestion that in Darier's disease, as in psoriasis, persistence of disease may require an intact papillary dermis.

Significance to Cancer Research:

1) Knowledge of the controls governing cell migration after injury are

essential to the understanding of malignant growth.

Proposed Course of Project:

1) Continued long-term observation of treated patients.

Publications:

1) Dellon, A.L., Chretien, P.B. and Peck, G.L.: Successful treatment of Darier's disease by partial thickness removal of skin. Plastic and Reconstructive Surgery 59:823-830, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3647-04 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Scanning and Transmission Electron Microscopy of Leukocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B. K. Wetzel Senior Investigator Derm NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 0.55	PROFESSIONAL: 0.05	OTHER: 0.05

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize definitively the origin, contents and fate of the characteristic granules of neutrophils, eosinophils, and basophils as an index of the development, function and fate of these cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To define the surface ultrastructure of leukocytes under a wide range of conditions and thereby provide fresh insight into the physiology of normal and leukemic cells; also, to characterize definitively the origin, contents, and fate of the characteristic granules of neutrophils, eosinophils, and basophils as an index of the development, function and fate of these cells.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Investigate the biology of cells already capable of forming cancer to determine the relationship of their growth and interaction with the host response.

Methods Employed:

Leukocytes from buffy coat and Ficoll-Hypaque gradient separations have been fixed in suspension and settled on or filtered onto a variety of substrates coated in various ways with poly-L-lysine -- a substance said to attach fixed cells firmly enough that they are retained in high yield through routine sample preparation for SEM. Retention was evaluated by visual or photographic comparison of the same fields before and after washing and/or dehydration and drying by the CO_2 critical point procedure.

Major Findings:

None of our approaches have succeeded in consistently retaining 90% or more of the leukocytes on the substrate through subsequent processing steps. We have made several attempts to repeat the original work, and have tried to improve the reliability of the method through the use of more likely substrates than glass, the use of poly-L-lysine of different molecular weights, more elaborate precleaning measures, attempts to derivatize the substrate, and efforts to improve the chemical and physical conditions for cell attachment. We must conclude that this popular method cannot be used under the assumption that it will produce consistently high yields of fixed cells settled from suspension. Therefore, our work on leukocyte topography must be done with the less popular but more reliable freeze-drying method developed in this laboratory.

Significance to Cancer Research:

The reactions of lymphocytes and macrophages to immune responses in malignant growth and therapy are largely dependent on cell surface phenomena and cell interaction -- both uniquely amenable to study by SEM and ancillary methods.

A thorough understanding of the origin and nature of leukocyte granules is necessary to define and interpret the differentiation of normal granulocytes, the cytopathology of chronic granulocytic leukemia and related disorders, and to help clarify the nature and functions of granulocytic infiltrates in skin and other tissues in malignant diseases.

Proposed Course of Project:

We will be engaged in a thorough description of the topography of peripheral leukocytes from normal humans obtained in representative yields. This approach will be extended to studies of hematopoiesis and the leukemias. A major study of leukocyte granulogenesis is planned employing quantitative autoradiography, cytochemistry and TEM. Light microscopic monitoring of preparative conditions and the dynamics of leukocyte adherence, motility, interaction, and growth will be continued with the use of thin perfusion chambers.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3648-05 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
T Cell Leukemias and Lymphomas: Morphologic and Functional Studies of the Neoplastic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.A. Lutzner	Senior Investigator	Derm NCI
OTHER:	R.L. Edelson	Associate Professor-Columbia Univ. College of Physicians & Surgeons	
	I. Green	Medical Officer, Research	I LI
	T. Ahmed	Bethesda Naval Hospital, Research Unit	
	S. Broder	Investigator	MET, NCI
	J. Whang-Peng	Section Head	MO NCI

COOPERATING UNITS (if any)
National Institute of Allergy & Infectious Diseases
Bethesda Naval Hospital, Research Unit

LAB/BRANCH
Dermatology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) To characterize the structure and function of the abnormal cells in the T cell leukemias. Precise identification of inflammatory cells in lesions. To study structure, function and oncological significance of the characteristic cell of the Sezary syndrome, and to study similar parameters in malignant cells of mycosis fungoides and related cancers. (1) Patients sought with Sezary syndrome and with mycosis fungoides. Specimens of blood, marrow, skin node, etc. will be obtained for study: (2) Tissue cells and buffy coat preparations will be Feulgen-stained and examined with a cytophotometer in order to detect presence of polyploid cells; (3) Tissue biopsies and buffy coat pellets will be examined with the electron microscope for morphologic identification of characteristic cells: (4) Chromosome analysis of abnormal tissue, marrow and blood cells will be made in order to determine if chromosomes are normal or abnormal in number and structure; and (5) Immunologic techniques will be used to determine whether Sezary cells belong to either B-or T-lymphocyte class. Frozen sections of tissue are layered with sheep erythrocytes containing on their surfaces moieties which will specifically interact with receptors on mononuclear cells. Histiocytes have receptor for IgC antibody and B cells have receptors for the third component of complement.

Project Description:

Objectives

1) To determine whether those neoplastic cells which preferentially infiltrate the skin share common features.

2) To relate the clinical findings in these patients to the distinctive properties of the neoplastic cells.

3) To establish more effective means of therapy of these disorders through exploitation of the distinctive properties of the neoplastic cells.

4) To use the homogenous cells found in these disorders as reagents for investigation of T cell function.

5) To isolate the product of the mixed leukocyte reaction locus.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 4.1: Investigate the biology of cells already capable of forming cancer to determine the relationship of their growth and interaction with the host response.

Methods Employed:

1) Tissues to be studied are sectioned in a cryostat, layered with sheep erythrocytes containing on their surface moieties, which will specifically interact with receptors on mononuclear cells, rinsed to remove nonadherent cells, fixed in glutaraldehyde, and stained with H and E to be viewed by light microscopy.

2) Tissue, cells and buffy coat preparations are Feulgen-stained and examined with cytophotometer in order to detect presence of polyploid cells.

3) Lymphocytes are grown in short term culture and the karyotype determined.

4) Lymphocytes and tissue were fixed and stained routinely for electron microscopy.

5) Lymphocytes are tested for responsiveness to the mitogens phyto-hemagglutinin, pokeweed and concanavalin A and for stimulating and responding capabilities in mixed leukocyte cultures.

6) Lymphocytes are tested for sheep erythrocyte receptors, complement receptors, Fc receptors, IgG receptors, surface immunoglobulin, and specific antigenic determinants.

7) Lymphocytes, fibroblasts and keratinocytes are tested for effector and target cell potential in cell mediated lympholysis reaction.

8) An antiserum specific for T cells has been produced and purified by absorption with B cells, red cells, and platelets. This antiserum may be used therapeutically in cases unresponsive to other more conservative measures.

9) Leukapheresis, a process by which blood is passed through a continuous flow centrifuge with white cells being selectively removed from the buffy coat, has been used in the therapy of one patient.

Major Findings:

1) T cells, of at least certain morphologic varieties, preferentially infiltrate the skin and spare the bone marrow.

2) If the Sezary syndrome is redefined as T-cell leukemia with associated erythroderma, we have now identified three morphologic variants.

3) Each patient in this expanded Sezary group differs from the other patients at the cellular level, using functional, antigenic and morphologic criteria. In addition, several patients had unique clinical features, i.e. Jin's inverse cyclicity and F.S.'s recurrent hepatic and renal infiltration.

4) The first case of lymphocytic leukemia with both B and T cell markers on the same cells was identified.

5) Review of the cumulative NIH experience with the Sezary syndrome revealed a remarkable lack of thymic infiltration at autopsy. This plus the identification of T cells in the skin of our more recent patients suggests that the skin may actually be playing a role in the aberrant differentiation of these abnormal cells.

6) An affinity of T cells for the skin is supported by our observation that the large majority of cells in mycosis fungoides, lichen planus, Mucha Habermann's disease, parapsoriasis en plaque and lymphomatoid papulosis also appear to be T cells.

7) One patient with T cell leukemia and tumor stage mycosis fungoides superimposed on a generalized erythroderma was treated with leukapheresis, a process by which leukocytes are selectively removed from the buffy coat in a continuous flow centrifuge, and the cutaneous lesions resolved. The patient returned 100 days later from California for a repeat of the leukapheresis with similar results.

8) Cells from one patient with the Sezary syndrome have been maintained in culture for one month and retain both their receptors for sheep

erythrocytes and their distinctive morphology.

9) An immunotherapy protocol has been devised and accepted for use. However, because of the relative success of our more conservative therapeutic approaches, immunotherapy has not yet been required. Other approaches include the present trial run of L-asparaginase which appears to be of at least limited efficacy.

10) Three patients with cutaneous T cell leukemia have now been treated by leukapheresis. One patient, who presented at the time of initial therapy, with tumor stage mycosis fungoides superimposed on a generalized erythroderma and with a lymphocytosis of more than 200,000 per cu mm has been adequately managed for the last 18 months by leukapheresis alone. Two other patients have also significantly benefited from leukapheresis. One patient is presently undergoing extracorporeal irradiation to determine whether this is a more efficient means of managing patients with lower peripheral blood lymphocyte counts.

11) Cells from one patient were hypotonically lysed following leukapheresis and membrane components were conjugated to a cyanogen bromide activated sephadex column. This column was subsequently used as an immuno-absorbent column to remove and then concentrate the anti-T cell activity and the active factors are themselves valuable reagents.

12) Serum from each of 6 tested patients with cutaneous T cell leukemia contained large amounts of macrophage inhibitory factor, which could be traced back to the leukemia cells. The serum of these patients therefore represent reservoirs from which this material can be isolated and characterized. Of clinical significance, the presence in the serum of a factor which has a profound effect on circulating monocytes may be at least in part responsible for observed anergy in the patients and their susceptibility to infection.

13) Patients with lymphomatoid papulosis were evaluated with regard to their clinical presentation, microscopic, ultrastructural and cell surface marker characteristics of their cutaneous infiltrates, their response to challenge with intradermal antigens, and the response of their peripheral leukocytes to mitogen and antigen stimulation.

Preliminary findings revealed that the infiltrating cells of lymphomatoid papulosis were thymus-derived lymphocytes and the nuclear irregularity of the cell of the cutaneous infiltrates was significantly greater than that of infiltrating lymphocytes of delayer hypersensitity skin reactions, with a pattern similar to that seen in the circulating lymphocytes of the Sezary syndrome.

Patients with lymphomatoid papulosis demonstrated normal reactivity to intradermally applied antigen and normal in vitro stimulation of lymphocytes by mitogens.

Chromosome studies were carried out on 11 NIH patients who had the diagnosis of Sezary syndrome. Heteroploidy, multiple markers including minute and ring chromosomes, and a lack of modality and clone formation, were the common chromosomal findings in this syndrome. Abundant spontaneous division of heteroploid cells in unstimulated peripheral blood cultures, a high percentage of heteroploid cells in stimulated culture, and finally, clone formation, are signs of a fulminant process and lead to the terminal phase of this disease. Early chemotherapeutic eradication of these heteroploid neoplastic cells would therefore be the treatment of choice in this disease.

Significance to Cancer Research:

By identifying the roles of mononuclear cells in defense against non-lymphoid cancer and accurately characterizing the types of lymphoid cells causing various lymphomas, a more rational approach to the treatment and prevention of these diseases is possible.

Proposed Course of Project:

Patient's with this syndrome will be referred to other branches of the Cancer Institute who have become interested in studying Sezary cells and treating Sezary patients.

Publications:

1) Green, I., Jaffe, E., Shevach, E., Edelson, R., Frank, M., Berard, C.: Determination of the origin of malignant reticular cells by the use of surface markers. Monograph - International Academy of Pathology. Eds. Williams & Wilkins, pp. 282-300, 1975.

2) Whang-Peng, J., Lutzner, M., Edelson, R. and Knutsen, T.: Cytogenetic studies and clinical implications in patients with Sezary syndrome. Cancer Research 38:861-867, 1976.

3) Broder, S., Edelson, R.L., Lutzner, M.A., Nelson, D.L., MacDermot, R.P., Durm, M.E., Goldman, C.K., Meade, B.D., and Waldmann, T.A.: Sezary syndrome: A malignant proliferation of helper T cells. J. Clin. Invest. 58:1297-1306, 1976.

5) Schein, P.S., MacDonald, J.S., and Edelson, R.: Cutaneous T-cell lymphoma. Cancer 38:1859-1861, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 03649-05 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Enzymatic Control Mechanisms in Melanogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	J. M. Nicholson	Post-Doctoral Fellow	Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has partially characterized the nature of tyrosinase, and determined the mechanism of normal latency of the enzyme, and its subsequent activation in vivo. The role of this enzyme in melanogenesis in normal tissue and the importance of the altered enzyme found in melanoma tissues is being studied.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To investigate the control mechanisms involved in regulating melanogenesis in a variety of normal and malignant tissues.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Tryosinase and other enzymic activities are investigated both by spectrophotometric and radioactive assay methodology. Tissues are dissected immediately before use in 0.1 M phosphate buffer, homogenized and fractionated into subcellular fractions by means of density gradient centrifugation and differential centrifugation. After solubilization with detergents, enzymes are further purified by polyacrylamide gel electrophoresis. Samples are then incubated with the appropriate substrates and controls. The production of melanin and other reaction products can be followed spectrophotometrically or by liquid scintillation counting of the newly formed radioactive melanin or byproducts.

Major Findings:

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in both murine and avian pigmentary systems, but not peroxidase, as has been suggested by some workers. It has always been a subject of dispute how tyrosinase, which can be easily demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated *in vivo* in mammalian systems and subsequently activated once *in situ* in the melanosome. In amphibians, the mechanism seems to be proteolytic activation; in mammalian and avian systems, however, proteolysis has no function in this regard. L-DOPA is the natural activator of the enzyme in vivo; we have tested over twenty L-DOPA analogs substituted in various positions, and determined the enzyme is extremely specific in its requirement for L-DOPA as a cofactor. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues.

Significance to Cancer Research:

In view of the differences noted between the regulation of tyrosinase in normal and melanoma tissues, we feel that at lease one of the primary differences between the melanogenic capabilities of these two tissues has been revealed. The fact that these controls are operational at the post-translational level is informative; whether other levels of cellular control mechanisms are affected by carcinogenesis remains to be investigated. It is hoped that further study of the cause of these enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Proposed Course of Project:

Differences in the control mechanisms over melanogenesis in normal and malignant melanoma tissues will continue to be investigated. The characteristics of enzymatic activity and control in various subcellular fractions are being studied. This project has been expanded to include characterization of similar metabolisms in normal and malignant human tissues.

Publications:

1) Eppig, J.J., Jr. and Hearing, V.J.: The bifunctional role of mammalian, avian, and amphibian tyrosinases in melanogenisis. Proc. IX Inter. Pigment Cell Conf. 3:82-88, 1976.

2) Hearing, V.J. and Ekel, T.M.: Mammalian tyrosinase: a comparison of tyrosinase hydroxylation and melanin formation. Biochemical Journal, 157:549-557, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3650-05 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Mammalian Melanosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. J. Hearing	Research Biologist	Derm NCI
OTHER:	J. M. Nicholson	Post-Doctoral Fellow	Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The abnormal proteins produced in malignant melanoma are being studied: these studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To characterize the structural and enzymatic composition of melanin granules throughout their development in vivo, both in normal and abnormal pigmentary systems.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Our research has been aimed primarily at characterizing the chemical composition of melanosomes in urine and human pigment systems, both in the normal as well as in melanoma tissues. Tissues are excised and homogenized in 0.25M sucrose, and the melanosomes are then isolated and purified by means of an extensive series of differential centrifugations. Granules thus isolated are solubilized in either sodium decyl sulfate, 8 M urea or Triton X-100, then examined with the aid of polyacrylamide gel electrophoresis. Computer analysis of the resulting protein separations allows the calculation of various physiochemical parameters of these macromulecules, including molecular weight, molecular size, valence and several other physical constants that are most useful in the comparisons of various proteins. Preparative polyacrylamide gel electrophoresis has enabled mg quantities of the proteins under study to be isolated in a pure state. This allows further characterization of these proteins with regard to their amino acid content, isoelectric point, molecular weight, and prosthetic group content.

Major Findings:

The melanosome in normal mouse tissue is composed of multiple proteins, many of which are loosely bound and easily extracted. These probably constitute the proteins of the limiting membrane of the organelle. Another protein is of lower molecular weight and is tightly bound to the granule, and probably constitutes the structural, fibrillar protein; it appears to be the protein which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomes are unique and are not found in normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of

isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, gly, ala, leu).

Significance to Cancer Research:

The observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. Melanomas are unusual among cancers since they do not result in complete differentiation of the affected melanocyte, but allow this cell's specialization, i.e. melanogenesis, to continue. These results indicate that although melanogenesis takes place, its metabolic pattern is abnormal.

There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanosomes. An analogous situation of altered proteins has been described in several other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. The similarity of these proteins suggests that the unique proteins present in the malignant melanocyte are an aberrant form of the analogous protein in the normal melanocyte, although the mechanism effecting this has not yet been elucidated.

Proposed Course of Project:

Since there are indications that the metabolism of melanogenesis in murine melanoma is aberrant, our research is ultimately intended to examine human melanomas, and attempt to characterize the level on which these disorienting control mechanisms operate. It is hoped that further insight into such controls, be they at the level of replication, transcription, translation, or post-translation, will provide clues as to the level on which carcinogenic information is expressed.

Publications:

1) Klingler, W.G., Montague, P.M. and Hearing, V.J.: Unique melanosomal proteins in murine melanoma. Proc. IX Inter. Conf. Pigment Cell Biol. 2:1-12, 1976.

2) Hearing, V.J., Klingler, W.G., Fkel, T.M. and Montague, P.M.: Molecular weight estimation of Triton X-100 solubilized proteins by polyacrylamide gel electrophoresis. Anal. Biochem. 72:113-122, 1976.

3) Klingler, W.G., Montague, P.M., Chretien, P.B. and Hearing, V.J.: Atypical melanosomal proteins in human malignant melanoma. Arch. Derm. 113:19-23, 1977.

4) Kerney, S.E., Montague, P.M., Chretien, P.M., Nicholson, J.M., Ekel, T.M., and Hearing, V.J., Jr.: Intracellular localization of tumor-associated antigens in murine and human malignant melanoma. Cancer Research 37:279-284, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 3652-04 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies on Mechanisms of Cellular Immunity in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.R. Levis	Senior Investigator	Derm NCI
OTHER:	A.M. Dattner	Instructor, Albert Einstein School of Medicine	IEP AIP

COOPERATING UNITS (if any)
Diabetes Branch, NIAMD, Reproduction Research Branch, NICHD

LAB/BRANCH
Dermatology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A better understanding of cell mediated immunity in man. Particularly, normal or abnormal immunologic mechanisms involved in epithelial and lymphoid malignancies, auto-immune disease, allograft rejection and allergic contact dermatitis. Characterization of the abnormal blastogenic responses found in certain diseases, particularly lymphoid malignancies. The necessity of both lymphocytes and macrophages in blastogenic responses in vitro has been elucidated and the lymphocyte has been identified as the cell which determines the immunologic specificity of the blastogenic response to antigens. A soluble mediator, released by macrophages, has been found to be an essential requirement for the blastogenic response to antigens and foreign tissues. Soluble mediators (lymphokines), released by various subpopulation of lymphocytes, have also been detected and studied at varying times following specifically induced cell-mediated contact sensitivity reactions. Our combined in vitro and in vivo controlled methods for the study of cell-mediated immunity in man, have allowed us to initiate basic immunopharmacology and immunogenetics approaches for the study of a variety of diseases and has already resulted in several practical diagnostic procedures. Additional studies promise a method for selecting effective immunotherapeutic agents against specific cancers.

Project Description:

Objectives:

1) To investigate the mechanisms concerned with the control of mitosis, differentiation, function, and potentialities of normal and pathologic cells, with particular reference to the lymphocyte and cells of the lymphoreticular system.

2) To investigate the functional and morphological interactions between lymphocytes and macrophages and among different populations of lymphocytes during in vitro immune responses related to delayed hypersensitivity, homograft reactions, and cell mediated allergic contact dermatitis.

3) To detect, characterize, and isolate biologically active substances, including soluble mediators of cellular immunity, produced in stimulated and unstimulated human leukocyte cultures. To study their effects modulation, and differentiation in vitro.

4) To develop an in vitro system for studying allergic contact dermatitis in humans including a method of monitoring immunotherapy of skin cancers with DNCB.

5) To develop a useful and sensitive histocompatibility test in humans.

6) To develop an in vitro model for the study of homograft rejection in humans.

7) To develop an in vitro system for studying immune reactivity to various skin cancers.

8) To determine the efficacy of 2,4 dinitrochlorobenzene (DNCB) on cutaneous malignancies and premalignant growth in subjects sensitized to DNCB.

9) To evaluate DNCB as a useful model for the study of immunotherapy of certain human cancers and possibly lead to additional immunotherapy studies.

10) To develop an in vitro model for studying and screening drugs which modulate cell mediated immunity in humans.

The Objective and Approach of the National Cancer Plan that this project most closely suppors is 6.1: Develop and utilize methodologies of immunology, chemotherapy, surgery and radiation (alone and in combinations) in the cure and palliation of cancer.

Material:

1) Lymphocytes and other peripheral blood cells are obtained from hospitalized patients and outpatients including patients with chronic lymphocytic leukemia, sezary syndrome, and other disease states.

2) Keratatome strips of human skin.

3) Excised tumors of human skin.

4) Sperm cells obtained from human ejaculates.

5) Fibroblasts, lymphoid cells from humans and other species maintained in tissue culture.

Methods Employed:

1) Human peripheral blood lymphocytes are cultured in vitro and stimulated to transform into large blastoid cells by factors such as phytohemagglutinin, antigens, and foreign tissue. This phenomenon of blastogenesis is evaluated by the morphologic and biochemical changes which the lymphocytes undergo. The mechanisms by which these blastogenic factors act and the culture conditions required for blastogenesis are determined.

2) Human peripheral blood leukocytes are separated into a glass-adherent cell population, composed largely of macrophages, and into a population consisting of small lymphocytes. By recombining such populations obtained from the same and from different individuals, the function of each population is determined. Interactions and contacts between cells are examined by light and scanning electron microscopic techniques, including methods for observing the same cells by both modalities.

3) A method has been developed for freeze storing leukocytes for subsequent studies of the blastogenic response to various antigens and mitogens.

4) A method has been developed for adapting the dinitrochlorobenzene contact sensitization test to the in vitro lymphocyte transformation system.

5) Methods have also been developed for freeze storing human spermatozoa with preservation of HLA-D (LD) antigens.

Major Findings:

1) Methods for obtaining 'purified' lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human

peripheral blood have been developed and analyzed. We have improved and utilized a very rapid and simple 5-minute centrifugation method using heparinized whole blood for obtaining a population of mononuclear leukocytes (lymphocytes and monocytes) with very few contaminating granulocytes. This mononuclear leukocyte population contains the two cell types required for the blastogenic response and provides cultures capable of responding with maximal responses to specific and non-specific blastogenic factors.

2) Human epidermal cells induce blastogenesis in allogeneic leukocytes. Human epidermal cells did not induce blastogenesis in autologous leukocytes nor in the leukocytes of a mixed leukocyte HL-A identical sibling pair. These results indicate that epidermal induced leukocyte blastogenesis is a measure of histocompatibility in humans comparable to a one-way mixed leukocyte reaction.

3) A method has been developed which permits positive light microscopic identification of individual leukocytes in a heterogeneous population by Giemsa staining while preserving delicate surface features for subsequent scanning electron microscopy of the same cells.

4) Isolation and partial characterization of a factor released by glass adherent phagocytic cells which is an essential requirement for the blastogenic response of lymphocytes to specific antigens, leukocytes, and phytohemagglutinin.

5) Isolation of a factor released in cultures containing lymphocytes of subjects sensitized to DNCB that will induce blastogenesis in leukocyte cultures from nonsensitized subjects.

6) Characterization of the abnormal blastogenic responses of lymphocytes from patients with chronic lymphocytic leukemia, sezary syndrome, and other related disease states.

7) Development of a method for monitoring the degree of sensitivity to DNCB in vitro during in vivo contact sensitization. This in vitro technique expands the usefulness of DNCB for the evaluation and study of cell mediated immunity, and offers a method for monitoring DNCB immunotherapy, an approach for the study of tolerance, and a controlled system for the study of the many antigen-released mediators of cellular immunity in man. Similar approaches with other contact allergens may offer a safe diagnostic test for allergic contact dermatitis.

8) Successful adaptation of the Dinitrochlorobenzene (DNCB) contact sensitization test to the in vitro lymphocyte transformation technique in humans.

9) Development of simplified methods for freeze storing human peripheral blood leukocytes.

10) Using the frozen leukocyte technology and the method for studying DNCB sensitivity in vitro we have demonstrated a temporal relationship between in vivo skin reactivity and in vitro specific lymphocyte transformation during a primary immune response.

11) Peak in vitro activity was observed two to four weeks following application of the primary sensitizing dose of DNCB and fell off to lower levels within two months of primary sensitization. These sequential studies of allergic contact dermatitis in humans are the first such studies of the kinetics of a primary cellular immune response using both in vivo and in vitro techniques.

12) Additional studies have demonstrated a quantitative relationship between the intensity and duration of allergic contact dermatitis to DNCB in vivo and the magnitude of specific lymphocyte transformation in vitro.

13) Demonstration that repeated challenge with DNCB boosts specific in vitro lymphocyte transformation.

14) Discovery of lymphokine activity in allergic contact dermatitis there is evidence that such lymphokines may be the mediators of cell mediated immunity.

15) Demonstration of the time course of release of lymphokine activity in human peripheral blood cultures.

16) Demonstration of reproducible dose-response curves for lymphokine activity. Such dose-response curves illustrate a sensitive quantitative assay for cellular immunity in vitro and offers a biological system with great potential for further characterization and isolating the mediators in cell mediated immunity.

17) The DNCB system offered the versatility that made it possible to show the lymphokines responsible for lymphocyte transformation in target leukocyte populations from nonsensitized subjects were as active on syngeneic (identical twins) leukocytes as on allogeneic leukocytes, indicating the observed activity was not due to histocompatibility factors.

18) It has recently been shown that lymphokine activity is absent during a primary immune response to DNCB and required one or more rechallenges before activity is detectable using the lymphocyte transformation system which suggests the soluble factors known as lymphokines, may represent an amplification of cellular immunity.

19) Successful adaptation of an automated microsystem for harvesting cells has enabled us to harvest cells in a rapid fashion.

20) The unique features of this project are: a) Both in vitro and

in vivo portions of this project are ethically feasible in humans which makes these studies more germane to human disease. b) Previous studies with microbial antigens have lacked effective control over exposure, in contrast to controlled DNCB sensitization. c) The assay is sensitive, specific and quantitative making it a valuable tool for measuring lymphocyte activation under a variety of experimental conditions.

21) Studies on soluble factors released by human lymphocytes (lymphokines) indicate that little or no lymphokine is released during an in vivo primary immune response to DNCB, but is produced in some subjects only after additional in vivo rechallenge. We interpret this to indicate that the lymphokine activity demonstrated in vitro may well indicate a bodily mechanism for amplifying the cellular immune response.

22) Recent studies in our laboratory indicate that an entire series of DNP containing amino acids induce varying degrees of lymphocyte transformation specifically in subjects topically sensitized with DNCB. The ability to stimulate with soluble DNP containing substances as well as with our previously developed particulate antigen offers great flexibility in our combined in vitro - in vivo system for investigating the cellular immune response in humans. Furthermore, these studies might prove to be of considerable importance in understanding the mechanism of triggering a specific cell mediated immune reaction.

23) Demonstration of the ability of sperm cells to induce allospecific lymphocyte transformation. We have previously demonstrated the ability of macrophages, certain lymphocytes, and human epidermal cells to induce allospecific lymphocyte transformation. Interestingly, studies in the mouse indicated that a product of the major histocompatibility complex has a tissue distribution similar to the cell populations we have found to induce allospecific lymphocyte transformation in humans. These findings could have important impact in diverse areas such as transplantation and reproductive biology. We are currently attempting to find out if our studies on contact sensitivity are related or in any way controlled by the major histocompatibility complex in humans. The combination of our histocompatibility and contact sensitivity studies may develop into a field of human immunogenetics with potential ramifications in a wide variety of disease states.

24) We have developed methods for freeze-storing human spermatozoa and preserving their ability to stimulate allogeneic lymphocytes. This methodology, which uncovers the important HL-A-D (LD) locus responsible for graft rejection allows a practical and superior approach to study of the genetic locus containing immune response genes associated with a variety of human diseases.

25) In collaboration with the Diabetes Branch we have discovered a new form of diabetes with a defect in the affinity of insulin-receptors.

Preliminary evidence suggests that this defect may relate to circulating plasma factors (Clin. Research - Abstract (in press) Manuscript in preparation).

26) We have developed a method (clonal priming) for defining specificity and cross reactivity at the T cell level. The clonal priming reaction offers a practiacl, ethical, in vitro, quantitative approach for systematic study of relationships recognized as foreign by immune cells. Concepts of cross reacticity, organ- and tissue-specific antigens, autoimmunity, tolerance and immunohomeostasis have been well developed. However, progress has been hampered by lack of an experimental approach to directly compare tissue, differentiation, tumour, microbial (including viral) and other environmental antigens at the level of cellular immunity. With clonal priming, it may now be possible to pinpoint agents implicated in a variety of inflammatory and neoplastic diseases in which cellular immune discrimination plays a role.

Significance to Biomedical Research and the Program of the Institute:

The lymphocyte is a cell involved in immune reactions which may be operative in the immune response to neoplastic tissue. Our studies concerned with blastogenesis and the control of cell division and differentiation of the lymphocyte may elucidate the cell's normal and abnormal functions in the immune response involved in the host's defense against cancer. These studies should also provide information concerning the control of cell differentiation and division.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

1) Levis, W.R., Dattner, A.M.: Clonal priming of human lymphocytes: Specificity and cross reactivity of cellular immune reactions. Lancet 1:875-877, 1977.

2) Levis, W.R. and Seidman, C.E.: Guest Editorial - HLA and other immunogenetic approaches to the study of diseases in man. Biomedicine, 26:5-8, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3654-04 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Applications of SEM to Soft Biological Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.K. Wetzel	Senior Investigator	Derm NCI
OTHER:	K. Sanford	Head, Cell Physiology & Oncogenesis	LB NCI
	C. Fox	Senior Investigator	LB NCI
	G. Jones	Microbiologist	LB NCI
	R. Tarone	Staff Fellow	B NCI

COOPERATING UNITS (if any)
Laboratory of Biochemistry, Biometry Branch, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.45	0.95	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experiential view of cell form and cell surface phenomena, and 3) an unusual potential for cytochemical studies.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experimental view of cell form and surface phenomena, and 3) an unusual potential for cytochemical studies.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.5: Study cell surface and cell membranes.

Methods Employed:

Tissue culture cells are grown as monolayers on glass coverslips, fixed by the addition of an equal volume of buffered 6% glutaraldehyde to the medium and processed appropriately for SEM.

The growth of Chinese hamster ovary cells in standard monolayer culture was recorded by phase contrast time lapse cinephotomicrography for two days through the addition of tritiated thymidine (^{3}H-TdR) 15 minutes before fixation. All cells in a predetermined area were systematically scanned, the samples were dipped in liquid emulsion, and the resultant autoradiographs of the same cells were examined and recorded by photomicrography.

Major Findings:

This study of mouse embryo cells represents the most rigorous topographic comparison between neoplastic and nonneoplastic cells to date. The reported correlation between numerous microvilli, blebs, or ruffles and neoplastic state in vitro is based on extraordinarily vigorous long-term neoplastic lines or on morphologically transformed variants, and thus could reflect their unusual growth properties rather than the neoplastic state. On the other hand, the cells in the present study were of common clonal origin with very similar histories in vitro and were compared with regard to tumorigenicity in the absence of deliberate carcinogenic stimuli without preselecting morphologically altered transformants. Under these conditions the neoplastic cell populations tended to appear less spread and more heterogeneous than the nonneoplastic cell populations, but showed no greater incidence of microvilli, blebs, or ruffles. These observations were also true among cells of comparable postmitotic time (determined by cinephotomicrography of the scanned cells), thus excluding the possibility that accelerated growth might obscure characteristic neoplastic cell form by reducing

the proportion of cells in the ostensibly villous G_1 stage of the cell cycle.

The widely held notion that the topography of cells in monolayer culture changes predictibly as they progress through interphase stages of the cell cycle has been based entirely on studies of synchronized cell populations that have not been controlled for the impact of forcible synchronization procedures on cell form. Cinephotomicrography reveals that under our conditions ^{3}H-TdR addition, fixation procedures, and processing for SEM do not appreciably alter CHO cell topography. Thus, the surface morphology of cells in S phase (identified by autoradiographic assay of ^{3}H-TdR incorporation into cells that have been scanned) can be distinguished from cells not in S phase without perturbing their growth. Our initial results clearly indicate that the S phase of the cell cycle has little or no influence on the topography of these cells in standard asynchronous monolayer culture.

Significance to Cancer Research:

SEM reveals fine surface structure with unparalleled three-dimensionality, depth of field, and sampling capability. As this approach is adapted to biological problems through experience with diverse tissues, cellular form, surface details and interactions can be better interpreted in normal and malignant tissues, as well as in experimental systems which help define immune responses, differentiation, and other processes fundamental to oncogenesis and its control. The tissue culture studies directly elucidate new aspects of tumor cell biology and transformation. The autoradiographic studies for the first time demonstrate that interphase stages in the cell cycle (skewed by neoplastic growth) may have little or no influence on the topography of cells in monolayer culture.

Proposed Course of Project:

To further develop and refine techniques of specimen preparation, analysis, and interpretation for SEM will be the goal of continuing work with a wide variety of tissues, experimental approaches, and instrumentation. Special emphasis will be accorded a search for novel cytochemical methods, especially with labels visualized in the backscatter mode, which are uniquely possible for this tool. Further collaboration in SEM, TEM and cytochemical studies of skin is under way with investigators in this branch and elsewhere.

Publications:

1) Wetzel, B., Sanford, K., Fox, C., Jones, G., Westbrook, E. and Tarone, R.: Topography of non-neoplastic and neoplastic cells of common origin. Cancer Res. 37:831-842, 1977.

2) Wetzel, B., Jones, G.M. and Sanford, K.K.: Cell cycle and topography in non-synchronized monolayers: the use of autoradiography and time-lapse for more rigorous SEM studies. Scanning and Electron Microscopy, 1977, Volume I, O. Johari, ed., IITRI, Chicago, Ill., pp. 545-552.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3656-04 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Steinert	Expert	Derm NCI
OTHER:	G.L. Peck	Senior Investigator	Derm NCI
	S. Yuspa	Section Head	DCCP NCI

COOPERATING UNITS (if any)

Experimental Pathology Branch, DCCP

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The seven polypeptide chains which comprise the subunits of the keratin filaments of normal bovine epidermis have been isolated and characterized. The unfractionated polypeptides or mixtures of as few as two polymerize in vitro into filaments which have the same physical and chemical structure as the in situ keratin filaments. The stoichiometry of these recombinations and the α-helical type X-ray diffraction patterns displayed by the filaments indicate the presence of a three-chain unit structure for the epidermal filament. Studies on the mechanism of polymerization in vitro and characterization of the α-helical regions of the filaments will provide further information on the ultrastructure of the filaments. The keratin polypeptide subunits of abnormal and malignant human epidermis are different from those of normal epidermis and work is in progress to define these differences. Normal mouse epidermal cells grown in cell culture synthesize the keratin filaments as well as a muscle actin-like protein. The appearance of these proteins in the cells grown in culture will be used as biochemical markers for studies of in vitro carcinogenesis. The actin-like protein is also present in a variety of human and mouse epidermal tumors and may be used as a marker for malignant tumors.

Project Description:

Objectives:

1) To characterize the ultrastructure of keratin filaments polymerized in vitro from the subunits of the filaments isolated from normal bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To investigate the biosynthesis of the keratin filament polypeptide subunits in cultures of normal mouse epidermal cells and to investigate the effects of various drugs and carcinogens on the cells.

4) To characterize the actin-like protein detected and isolated from normal mouse epidermal cells grown in culture and from mouse and human epidermal tumors. Attempts will be made to develop specific immunofluorescent- or radio-immune-assays for the actin-like protein.

Major Findings and Methods Employed:

1) The seven polypeptide chains which comprise the subunits of the keratin filaments of normal bovine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 Å wide and up to 40 um long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptide are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the X-ray diffraction analyses.

2) Studies on the mechanism of filament assembly indicate that polymerization is time and protein concentration dependent and follows an ordered biphasic cooperative process. This involves the initial rate-limiting formation of a "nuclear" particle followed by a rapid aggregation of it into filaments. The nuclear particle is a hexamer, as determined by mathematical models of light-scattering data, and has dimensions of 40Å X 1000Å in the electron microscope. This hexamer probably consists of a pair of triple chain structural units and may be a structural "building block" of the filament. In support of this, filament formation appears in the electron microscope to involve (a) apparent twisting of two or three aligned 40Å units to form an 80Å particle; and (b) end-to-end joining of either the 40Å units or 80A

particles. More extensive studies on these events are in progress.

3) The X-ray diffraction data implies the filaments certain region of coited-coil α-helix. These regions have been isolated by selective digestion with trypsin and appear in two forms: All of the helix of the filaments can be accounted for by (a) particles 20Å X 180Å, 95% α-helix, of 40,000 molecular weight, which can be denatured with SDS into chains of about 14,000 molecular weight. Therefore the α-helix of the filaments consists of discrete regions 200Å long of triple chain coiled coil α-helix. (b) particles present during the early stages of digestion are 20Å x 400 Å, 75% α-helix, of 110,000 molecular weight and can be denatured with SDS into chains of about 35,000 molecular weight. These presumably consist of two helical regions of (a) separated by about 40Å of non-helix. The properties of these fragments provides information on the alignment and staggering of the polypeptides in the filaments. Further information on the filament structure will involve detailed characterization of these trypsin fragments and of CNBr and NBS peptides derived from the intact polypeptide chain.

4) Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr peptides of the proteins obtained from normal and abnormal epidermis.

5) Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins are being isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogenesis. The keratin proteins provide a most satisfactory biochemical marker for following transformation by carcinogens in vitro. Initially specific antibodies to the keratin proteins will be elicited in rabbits for immunocytochemical assay purposes. In addition, the cells in culture also produce an actin-like protein and this is also of potential use for monitoring carcinogenesis in vitro.

6) The synthesis of the keratin filament subunits by the mouse epidermal cells implies the presence within the cells of the complete protein biosynthetic apparatus, and this system is therefore potentially very useful for studying control of differentiation in the epidermis. This is of importance as there are a number of diseases of the epidermis which may be attributed to disorders of the differentiative processes of the tissue.

7) A variety of human and experimentally-induced mouse epidermal tumors (carcinomas and papillomas) also contain large amounts of the actin-like protein. Because this observation has great potential as a marker for malignancies, the actin protein has been isolated and attempts will be made to prepare an antibody for a specific immuno-fluorescent- or radioimmuno-assay procedure. The large amounts of actin protein should provide a suitable biochemical marker for studies of the malignancies.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only because of the quantitative importance of this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of the keratin subunits in a well defined cell culture system will facilitate studies of cacinogenesis in vitro. In addition, the finding of large amounts of actin protein in epidermal tumors, and the development of suitable immunoassay procedures for it is of potentially great significance.

Proposed Course of Project:

Studies on the abnormal (benign and malignant) tissues will be done in collaboration with Dr. Gary Peck. The cell culture studies will be done in the Experimental Pathology Branch in collaboration with Dr. Stuart Yuspa. The biochemical, biophysical and electron microscopic studies of the structure of normal bovine epidermal keratin filaments will be done in this laboratory.

Publications:

1) Steinert, P.M., Peck, G.L., Yuspa, S.H., DiPasquale, A., and McGuire, S.: The isolation of an actin-like protein from epidermal tumors and cultured epidermal cells. J. Invest. Dermatol. 66: 276, 1976.

2) Steinert, P.M. and Gullino, M.I.: Bovine epidermal keratin filament assembly in vitro. Biochem. Biophys. Res. Commun. 70:221, 1976.

3) Steinert, P.M.: The mechanism of assembly of bovine epidermal keratin filament in vitro. In Biochemistry of Cutaneous Epidermal Differentiation. Seiji, M. and Bernstein, I.A., editors. Univ. of Tokyo Press, Tokyo, 1977, pp. 444-466.

4) Steinert, P.M., Idler, W.W. and Zimmerman, S.F.: The self-assembly of bovine epidermal keratin filament in vitro. J. Mol. Biol. 108: 547-567, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3657-03 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.I. Katz	Senior Investigator	Derm NCI
OTHER:	H. Yaoita	Expert Consultant	Derm NCI
	T.J. Lawley	Clinical Associate	Derm NCI
	G. Stingl	Visiting Fellow	Derm NCI

COOPERATING UNITS (if any)

Metabolism Branch, NCI, LCI, NIAID

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called autoimmune phenomena. We have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have centered around the mechanisms of tissue destruction, ultrastructure, and ultrastructural localization of antibodies. Gastrointestinal abnormalities, similar to those seen in gluten sensitive enteropathy, occur in dermatitis herpetiformis and are under active study. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of the effect of sulfones on neutrophilic exudative disorders. As sulfones dramatically alleviate dermatitis herpetiformis and erythema elevatum diutinum we are using animal models of inflammation and serum and blister fluids from patients to study their effect on complement activation, deposition and neutrophilic chemotaxis. Alterations in collagen synthesis in inflammatory disease and identification of alloantigens on epidermal cells are other integral parts of the overall program.

Project Description:

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine whether or not genetic factors are important in the developments of these diseases.

4) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

5) To identify the chemical characteristics of the antigen which is in antigen-antibody complexes.

Material:

Skin biopsies, either punch of Castroviejo keratome slices will be used. Also the small intestine of patients with dermatitis herpetiformis will be studied in order to determine their binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immunochemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays.

Major Findings:

1) All of 51 patients with dermatitis herpetiformis have *in vivo* bound IgA and various complement components in their skin.

2) The exact location of these antibodies has been sought and found to localize ultrastructurally in 1) clumps in the dermis, 2) associated with anchoring fibrils in the dermis or 3) along the lamina lucida of the basement membrane.

3) The early ultrastructural destruction of the skin in these patients has been demonstrated to correlate well with the antibody localization.

4) The sera of a small number of these patients contain circulating

antibasement membrane antibodies of the IgA class.

5) Almost all patients with dermatitis herpetiformis have morphologic and enzymatic evidence of gastrointestinal abnormalities very similar to those seen in patients with gluten-sensitive enteropathy.

6) In some patients it is possible to not only reverse the gastrointestinal abnormalities but to reduce or completely eliminate their requirement for sulfones by strict adherence to a gluten-free diet.

7) 85% of 51 patients with immunologically proven dermatitis herpetiformis have HLA-B8 as compared to 20% of controls.

8) All patients with dermatitis herpetiformis have a specific antigen on their B-lymphocytes. This antigen is present in a small percentage of the normal population.

9) Sulfones dramatically benefit patients with erythema elevatum diutinum.

10) By studying eight patients with herpes gestationis with immunologic methods we have been able to identify the serum factor responsible for deposition complement at the skin basement membrane zone, the site of earliest destruction.

11) Ultrastructural localization of complement correlates well with the earliest destruction seen in the electron microscope in herpes gestationis and further strengthens the concept that immunologic mechanisms play an important, if not essential, role in the pathophysiology of this disease.

12) The sera of patients with vitiligo associated with multiple endocrinopathies contain antibodies to melanin producing cells.

13) Langerhan cells which comprise a small population of epidermal cells, bear C3 and Fc receptors as well as alloantigens which are Ia associated.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and further current concepts in self-recognition. Pemphigus is associated with thymoma and myasthemia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions. Specific anti-collagen antibodies are being used to identify the stroma of various tumors, both human and murine.

Proposed Course of Project:

Outlined above.

Publications:

1) Katz, S.I., Hertz, K.C., Yaoita, H.: Herpes gestationis. Immunopathology and characterization of the HG factor. J. Clinical Invest. 57:1434-1441, 1976.

2) Hsueh, A.J.W., Dufau, M.L., Katz, S.I., Catt, K.J.: Gonadotropin receptors. Immunofluorescent labeling in enzyme dispersed intestitial cells. Nature 261:710-712, 1976.

3) Yaoita, H., Gullino, M., Katz, S.K.: Herpes gestationis ultrastructural localization of in vivo bound C3 and ultrastructure. J. Invest Derm. 66:383-388, 1976.

4) Katz, S.I., Hertz, K.C., Rogentine, G.N., Strober, W.: The association between HLA-B8 and dermatitis herpetiformis in patients with IgA deposits in skin. Archives of Dermatology 113:155-156, 1977.

5) Hertz, K.C., Katz, S.I., Maize, J., and Ackerman, A.B.: Herpes gestationis - A clinico-pathologic study. Arch. Derm. 113:155-156, 1977.

6) Yaoita, H. and Katz, S.I.: Immunoelectronmicroscopic localization of IgA in skin of patients with dermatitis herpetiformis. J. Invest. Dermatol. 67:502-506, 1976.

7) Hertz, K.C., Crawford, P.S., Chez, R.A., and Katz, S.K.: Herpes gestationis - an update. Obstetric and Gynecology. (In press).

8) Katz, S.K., Hertz, K.C., Crawford, P.S., Gazze, L.A., Frank, M.M., and Lawley, T.J.: Effect of sulfones on complement deposition in dermatitis herpetiformis and on complement mediated guinea pig reactions. J. Invest. Dermatol. 67:688-690, 1976.

9) Hertz, K.C., Aaronson, C. and Katz, S.I.: Juvenile dermatitis herpetiformis - an immunologically proven date. Pediatrics (In press).

10) Yaoita, H., Hertz, K.C. and Katz, S.I.: Dermatitis herpetiformis: Immunoelectronmicroscopic and ultrastructural studies of a patient with linear deposition of IgA. J. Invest. Dermatol. 67:691-695, 1976.

11) Hertz, K.C., Gazze, L.A., and Katz, S.I.: Immunofluorescent localization of basement membrane in lesions of dermatitis herpetiformis. J. Invest. Dermatol. 67:683-687, 1976.

12) Katz, S.I., Gallin, J.K., Hertz, K.C., Fauci, A.S. and Lawley, T.J.: Erythema elevatum diutinum: Skin and systemic manifestations,

immunologic studies, and successful treatment with dapsone. Medicine (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3658-03 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Search for Animal Models for Genetic Skin Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.A. Lutzner	Chief	Derm NCI
OTHER:	R.L. Kent	Biologist	Temple University Graduate School of Pharmacology
	K. Smith	Geneticist	R VR
	C.T. Hansen	Geneticist	R VR

COOPERATING UNITS (if any)

Division of Research Services, Veterinary Resources Branch

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been discovered that the vibrissaeless rat blisters because of a suprabasilar adhesion defect; that the exfoliative mouse suffers a transient purulent conjunctivitis, exfoliates and may die. Survivors continue to have intermittant hair loss; masked rats have blepharitis accompanied by alopecia. Pasteurella organisms appear to be playing a role. The motheaten mouse has been found to have a hyperimmunoglobulinemia and usually dies of an autoimmune disease. Germfree environment has no effect on prolonging survival. An attempt has been made to classify and summarize genodermatoses involving neoplasia.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To seek out animal models for human genetic skin diseases. Such mutations have occurred spontaneously or been produced in various animal production laboratories; some in our own animal laboratories at NIH.

Materials:

1) The Vibrissaeless (vb) rat, an autosomal recessive mutation, produced by multiple generation X-irradiation.

2) A mutation in the mouse occurring spontaneously called (me) which appears to have an immunological abnormality manifested by runt syndrome and early death.

3) A mouse mutant, which loses its hair in peculiar pattern and develops an inflammatory disease of the eyes.

4) A rat mutant in which the animals' eyes and snout are involved in an inflammatory disease.

5) A mutant mouse, in which the immunologic, endocrinologic and neurologic system are all involved.

Methods Employed:

Animals are bred either by heterozygote or homozygote matings. Some of the animals are kept in germ-free conditions, especially those that present immunologic defects. Tissues from these animals are studied by light and electron microscopy, by immunofluorescence, and by chemical and microbiological techniques.

Major Findings:

1) In the vibrissaeless rat it has been found that animals lose their hair at an early age and develop both spontaneous and traumatic blisters. Blisters can be produced by rubbing the skin. Histologically, separation occurs above the basal cell layer; therefore this is a suprabasalar blistering disease. Although there are several human diseases which resemble this defect, the rat does not appear to be an exact model for any known human genetic disease. Immunofluorescent studies have shown that this is not similar to pemphigus; collagen and collagenase studies have been normal; and animals have been found not to respond to systemic steroid therapy. The biochemical defect which is occurring in these rats has not been uncovered as yet.

2) The me mouse: Since these animals die at a very early age, detailed

immunologic studies have been impossible. Some of the animals have small thymuses and absent Pyer's patches. It has been found that unlike the nude mouse whose survival improves in a germfree environment this environment has no effect on the me mouse's survival.

3) The ex mouse: A spontaneous autosomal recessive mutation has been named the exfoliative mouse (genotype ex/ex). Exfoliative mice suffer a transient purulent conjunctivitis in their 3rd week and an exfoliative skin disease in their 4th week. Gram-negative bacilli are present in blood and cerebrospinal fluid during the conjunctivitis stage, and in skin during the exfoliative period. The mutant, whose exfoliative skin resembles the skin in human lamellar ichthyosis, can be differentiated from the ichthyotic mouse mutant.

4) The Masked rat: Two organisms have been isolated from the urine and eyes of these rats. They are both pasturella organisms and Koch's postulates have been satisfied in showing that these organisms are etiologically involved in producing the scaling inflammatory disease which occurs on the eyes and the snouts. The genetic basis of the susceptibility to these organisms has not yet been determined.

5) The Lethargic mouse: This is a mouse which has neurologic, endocrinological and immunologic abnormalities. No explanation has yet been found.

Significance to Biomedical Research and the Program of the Institute:

Many genetic diseases of the skin occur in which there is no knowledge of cause or treatment. These diseases are rare and experimentation is not easily performed on humans; therefore, it is of interest to try to develop or discover animal models for these diseases in the hope that when such animals have been found it will be possible to find the cause and treatment for these genetic abnormalities which would be hopefully translatable to human disease.

Proposed Course of Project:

Continuation of research as indicated.

Publications:

1) Kent, R.L., Lutzner, M.A. and Hansen, C.T.: Masked rat: An x-ray induced mutant with chronic blepharitis, alopecia and pasteurellosis. J. Heredity, 67:3-5, 1976.

2) Kent, R.L., Lutzner, M.A. and Smith, K.P.: Skin exfoliation and purulent conjunctivitis in a new mutant - the exfoliative mouse. J. Invest. Dermatol., 66:248-251, 1976.

3) Lutzner, M.A., Hansen, C.T.: Motheaten: An immunodeficient mouse with markedly less ability to survive than the nude mouse in a germ-free environment. J. Immunol., 116:1496-1497, 1976.

4) Lutzner, M.A.: Monograph: The Genetics of Human Cancer. Edited by J.J. Mulvihill, R.W. Miller, and J.F. Fraumeni, Jr., National Cancer Institute. Nosology among the neoplastic genodermatoses, Vol. 3, pp. 145-167, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 3659-03 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Therapy of Skin Cancer and Disorders of Keratinization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
OTHER:	F.W. Yoder	Clinical Associate	Derm NCI
	M. Pandya	Visiting Fellow	Derm NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oral 13-cis retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer and disorders of keratinization.

Agents:

1) 13-cis retinoic acid, 10 mg and 20 mg tablets.

Material:

1) Ichthyosis
   a) lamellar ichthyosis
   b) X-linked ichthyosis
   c) ichthyosis vulgaris
2) Darier's disease
3) Psoriasis
4) Keratoderma palmaris et plantaris
5) Pityriasis rubra pilaris
6) Nevus comedonicus
7) Basal cell carcinoma
8) Nevoid basal cell carcinoma syndrome
9) Xeroderma pigmentosum with cutaneous carcinoma

Methods Employed:

1) Disorders of Keratinization
   a) Oral 13-cis retinoic acid will be given for 4 months beginning at 1 mg/kg/day. The dose will be gradually increased (up to 6 mg/kg/day) to tolerance.

2) Skin Cancer
   a) Patients must have pathologic confirmation of the clinical diagnosis.
   b) They must have normal hematologic, hepatic, and renal function.
   c) Lesions on eyelids and canthi are not included.
   d) Patients are cautioned to avoid sun.
   e) Laboratory tests are done before, during, and 6 weeks after therapy.
   f) Serial photographs are taken.

Major Findings:

Sixty-four patients with treatment-resistant disorders of keratinization were treated with oral 13-cis retinoic acid, a synthetic stereoisomer of retinoic acid, for one to 29 weeks. The dosage varied from 0.5 to 6.0

mg/kg/day. The median patient received 2 mg/kg daily for 8 weeks. Excellent responses have been observed in patients with congenital ichthyosiform erythroderma, lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris, and cystic acne. However, patients with epidermolytic hyperkeratosis have responded only partially, and patients with psoriasis, nevus comedonicus, X-linked ichthyosis, and Netherton's syndrome have either not responded or have worsened. One patient with the nevoid basal cell carcinoma syndrome improved considerably with marked reduction in size of his skin tumors after therapy with oral 13-cis retinoic acid. Commonly observed side effects included cheilitis, facial dermatitis, xerosis, skin fragility, and conjunctivitis. The erythrocyte sedimentation rate was elevated in 7 patients; one patient had a temporary borderline elevation of his SGPT; no other abnormal laboratory tests were noted in this group of patients. The mechanism by which this synthetic retinoid alters these disease states is not clear but may be related to the observed ability of vitamin A to affect glycoprotein synthesis and epithelial differentiation.

Significance to Cancer Research:

a) These are exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer. Skin cancer should prove to be a valuable lesion for the screening of these agents because the tumors are observable, measurable, and therefore can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

b) The profound beneficial effect of 13-cis retinoic acid in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelia, could be successfully treated with the synthetic retinoids. These patients, who have successfully responded to 13-cis retinoic acid, may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Proposed Course of Project:

1) Continued treatment of patients with basal cells and disorders of keratinization with 13-cis retinoic acid (RO-43780).

2) Treatment of disorders of Keratinization with oral RO 10-9359 and topical RO 11-1430).

Publications:

1) Peck, G.L. and Yoder, F.W.: Lamellar ichthyosis treatment of

keratinizing and other dermatoses with an oral synthetic retinoid. Lancet ii:1172, 1976.

2) Peck, G.L. and Yoder, F.W.: Treatment of disorders of keratinization with an oral stereo isomer of retinoic acid. In The Ichthyoses (R. Marks, editor) MTP Press, Ltd., Lancaster, Fngland 1977 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CR 3660-02 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Transfection of Viral DNA of C-Type RNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. R. Lowy Senior Investigator Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A sensitive, quantitative assay has been developed to study the biologic effects of the DNA viral intermediate and provirus of mammalian C-type RNA viruses. This procedure is being used to assess the ability of DNA to be infectious and/or to induce cellular transformation. Optimal conditions for extraction and titration of viral-specific DNA have been defined. The effect of chemical and physical treatment on virus expression will be evaluated. Differences between unexpressed and expressed endogenous and exogenous viral genomes are under study.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To develop a sensitive quantitative biologic assay of viral-specific DNA (transfection) from C-type viruses.

2) To determine the effect of a variety of physical and chemical treatments of the transfecting ability of viral-specific DNA.

3) To determine the physical differences between unexpressed and expressed endogenous DNA, since transfection of expressed viral DNA is more easily accomplished than that of unexpressed viral DNA.

4) To define the smallest size of cellular DNA which can yield infectious virus.

5) To investigate the mechanism of activation of endogenous C-type viruses by halogenated pyrimidines.

6) To determine if transfection by the DNA viral intermediate and/or the DNA provirus is sensitive to the murine cellular Fv-1 gene, which restricts the replication of some classes of murine C-type viruses.

7) To define that the portion of the sarcoma virus genome which induces cellular transformation.

8) To utilize transfection as a method to detect portions of viral genomes in tumors, including those of human origin.

Methods:

1) For the isolation of provial genomes, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification.

2) For isolation of the DNA viral intermediate, DNA is purified by the Hirt procedure.

3) Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie.

4) Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of actinomycin D.

5) Virus is measured either by the XC plaque test of Rowe et al. or by focus-induction.

6) Nucleic acid hybridization between viral probes and cell DNA is carried out in liquid and analyzed either by S_1 nuclease or hydroxyapatite chromatography.

Major Findings:

1) A sensitive quentitative 10 day transfection assay for murine sarcoma and leukemia viruses have been developed for use with unintegrated and integrated viral DNA.

2) The efficiency of transfection depends on several critical parameters: size of infectious DNA, size of carrier DNA, concentration of DNA, cell density, pH of precipitation medium, and the use of dimethylsulfoxide.

3) With this technique, DNA can be titrated almost as easily as intact virus.

Significance to Cancer Research:

1) C-type viruses have been implicated in tumors of a wide variety of species, including man. Correlation of biologic and biochemical aspects of viral specific DNA will be of considerable help in elucidating the physiology of mammalian C-type viruses.

2) Since ease of activation of endogenous C-type viruses by IUdR appears to correlate with their ease of spontaneous activation, elucidation of the mechanism of activation would probably have relevance to spontaneous viral activation and perhaps to chemical carcinogenesis as well.

3) Transfection represents a technique which may be able to detect biologic activity of portions of viral genomes. Since some tumors in man may be induced by replication of defective viral genomes, this approach may be suitable for their detection.

Proposed Course of Project:

Continuation with attempted fullfillment of stated objectives.

Publications:

In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 3661-02 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Human Papilloma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. R. Lowy Senior Investigator Derm NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A combined biologic, biochemical, and serologic approach is being utilized to study the papilloma viruses associated with human warts. A radioimmune assay for viral antigen has been developed and is being refined. Purification of viral DNA will be accomplished for nucleic acid hybridization studies. These procedures will be used to monitor efforts to propagate virus in tissue culture as well as to evaluate the relatedness of virus in various types of warts. The relationship to other papova viruses will be explored. Skin from a variety of disorders of epidermal proliferation, including cancers associated with epidermodysplasia verruciformis, will be evaluated for the presence and expression of papilloma virus genetic material. Other malignant tissue will be studied virologically.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To develop a radioimmune assay for wart viral protein(s).

2) To develop a nucleic acid hybridization procedure for wart viral DNA.

3) To propagate wart viruses in tissue culture.

4) To measure the relatedness of viruses of warts from different patients, including those from patients with epidermo displasia verruciformis (EDV), laryngeal papillomas, condyloma accuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

5) To look for wart virus specific nucleic acid and/or antigen in skin cancers of EDV, kerato-acanthomas, and other tumors.

6) Relatedness of wart virus to other papova viruses.

Methods:

1) Purification of wart viruses from warts by CsCl density gradient centrifugation.

2) Isotopically labelling viral antigen and viral DNA with ^{125}I for conducting radioimmune assay and nucleic acid hybridization studies.

3) Purification of viral antigen by SDS gel electrophoresis and alkaline degredation.

4) Raise antibodies to wart virus in rabbits.

5) Isolation of viral DNA by ethidium bromide cesian chloride density, gradient centrifugation.

6) Restriction endonuclease digestion of viral DNA.

Results:

1) Viruses from plantar warts show at least 2 different restriction enzyme digestion patterns.

Significance to Cancer Research:

Warts represent a viral induced tumor of man. A closely related virus, the Shope papilloma virus of rabbits, will induce benign tumors in some rabbits of malignant tumor in others. The squamous cell cancers of patients with EDV may be induced by wart viruses, as may tumors of

other patients. The development of these reagents will not only permit the screening of tumors for wart viral material, they will also facilitate attempts to propagate the virus in tissue culture. Assuming that the analogy with the Shope papilloma virus is correct, propagation of the virus in tissue culture would facilitate the investigation of the molecular mechanisms which result in a benign proliferation of cells in some instances and malignant transformation in others.

Proposed Course of Project:

Continuation with attempted fulfillment of stated objectives.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 CB 3662-02 D

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Factors Involved in the Elimination of Superficial Mycotic Infection in Guinea Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. A. Lutzner	Senior Investigator	Derm NCI
OTHER:	A. D. Hernandez	Clinical Associate	Derm NCI
	J. E. Bennett	Senior Investigator	NIAID

COOPERATING UNITS (if any)
National Institute of Arthritis and Infectious Diseases

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A sensitive assay of fungal growth is being developed to study the possible factors involved in the limitation and elimination of superficial mycotic infections in guinea pigs, with emphasis being placed on possible role of cellular immunity and non-specific serum inhibitors.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) Quantitation of fungal spore germination using radiolabeled precursors.

2) Evaluate possible factors involved in limitation and elimination of superficial mycotic infection

a) epidermal turnover rate
b) delayed hypersensitivity and lymphokines
c) serum inhibition factors
d) non-specific products of inflammation

Methods:

1) Radiolabel defined culture media used to grow spores which are germinating - counts are correlated with visual quantitation of increase in size of hyphal element.

2) Guinea pigs will be infected with T. Mentagrophytes and course of infection monitored via skin biopsy and skin test antigens.

3) Sera from guinea pigs infected with T. Mentagrophytes and those in addition challenged with BCG will be studied for inhibition against T. Mentagrophytes and other fungi.

4) Lymphokines from guinea pigs stimulated by specific and non-specific antigen (Ag) will be assessed as to their ability to inhibit fungal growth.

5) Study effects of increasing epidermal proliferation on elimination of infection.

Major Findings:

Investigation of a soluble and particulate antigen of T. mentagrophytes showed these antigens were capable of eliciting erythema and induration 24 hrs. after being injected intradermally into guinea pigs sensitized with CFA-T. mentagrophytes or infected with T. mentagrophytes but no reactions in control animals. Skin tests were negative at 4 hrs. in all animals tested.

Both soluble and particulate antigen were capable of eliciting lymphocyte transformation in animals sensitized or infected with T. mentagrophytes but not in control animals. Particulate antigen is approximately 4 times more potent than soluble antigen in eliciting lymphocyte transformation.

Infections were consistently established using a T. mentagrophytes spore suspension placed under occlusion for 24 hrs. Infections ran a defined course. In normal non-manipulated guinea pigs no evidence of infection was noted until day 4-6 when mild erythema began to develop, lesions reached a maximum in size, crusting and hair loss at day 10-13, and animals were clinically and microbiologically cured by day 35-38.

Reinfection of these animals 10 days after they were spontaneously cured showed that erythema at infection site developed by day 2 and maximum size of infection site was achieved on day 7-9 and all animals were mycologically clear on day 17-18.

Infections were also established in animals sensitized with CFA-T. mentagrophytes and CFA alone. Animals which received only CFA showed erythema at infection site beginning on day 4-5 maximal size of lesion, scab and hair loss at days 10-12 and cleared on days 36-37. Guinea pigs sensitized with CFA-T. mentagrophytes showed erythema at infection site beginning on days 2-3, reached maximum intensity at days 7-9 and were clear by days 24-26.

Significance to Cancer Research:

Patients with immunosuppression (natural or induced) are susceptible to malignancy, as well as to bacterial, fungal and viral infections. Study of immune system from the point of view of mycotic infections, might provide insight into mechanisms involved and suggest treatment for such infections.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 3663-01 D

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Tumor virus expression in vitro and in vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.R. Lowy Senior Investigator Derm NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A flat mink cell line which contains the defective Maloney murine sarcoma viral genome (S^+L^- mink) becomes transformed upon superinfection with a helper virus. In the absence of helper virus, dexamethasone (10^{-8}M) treatment of S^+L^- mink induces a morphologically similar transformation. Removal of dexamethasone is followed by loss of transformation. The ability of other steroid hormones to induce this change parallels their glucocorticoid activity. By immunofluorescence, the transformed cells contain markedly increased levels of viral antigen. Transformation is accompanied by an increase in both Maloney sarcoma viral RNA and murine p30; these parameters fall to pretreatment levels following removal of glucocorticoid. No intact virus has been demonstrated. The parental mink cell line from which the S^+L^- mink was derived does not show these dexamethasone induced changes. These observations represent the first glucocorticoid dependent transformation system and the first documented increase in a C-type viral RNA by glucocorticoids. The results suggest that the glucocorticoid induced transformation is mediated through induction of the sarcoma viral genome.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To evaluate the effect of cell differentiation, primarily by chemical or hormonal treatment on RNA tumor virus expression and transformation in cells in tissue culture.

2) To evaluate the effects on hormones and other chemically endogenous and exogenous virus expression.

3) To gain insight into how the state of differentiation of a cell regulates tumor virus expression.

4) To improve our understanding of the development of sarcoma viruses.

Methods:

1) Treatment of cells or animals with hormones, other chemicals, and RNA tumor viruses.

2) Detection of virus expression biologically by XC plaque test and focus induction.

3) Radioimmune assays for viral antigens.

4) Nucleic acid hybridization for viral nucleic acids.

Results:

1) An untransformed mink cell which contains the Moloney murine sarcoma viral genome will undergo transformation on treatment with physiologic concentrations of dexamethasone or other glycocorticoids. The extent of transformation correlates with the levels of sarcoma specific viral RNA and with viral p30 protein. Transformation depends on the continued presence of glycocorticoid, since its removal results in loss of transformation and a decrease in viral expression to pretreatment levels.

2) In collaboration with Dr. N. Teich (Imperial Cancer Research Fund, London), several mouse teratoma cell lines have been examined for their ability to be infected by a variety of viruses. In the undifferentiated state, these cells were permissive for the replication of all viruses tested except for murine leukemia viruses. After differentiation, a cell line became permissive for Moloney leukemia virus. Nucleic acid hybridization studies indicate that the block to viral expression occurs after synthesis of proviral DNA, since as much viral DNA is found in the non-productively infected undifferentiated cells as in the productively infected differentiated cells. The block is probably at

the level of transcription, since viral RNA was not detected in the undifferentiated cells.

Significance to Cancer Research:

The ability of hormonal manipulation to affect tumor development has been well documented both in man and animals. Under certain conditions, hormones may also affect tumor virus expression. The $S^{+}L^{-}$ mink system represents the first hormone responsive system where transformation appears to depend on tumor virus expression. It should permit probing of the mechanisms by which steroid hormones might affect the development of cancer.

The issue of how cells control gene expression is central to the problem of the cancer cell. The teratomas provide a system where virus replication is clearly regulated by a cellular function whose expression depends on the differentiated state of the cell. Exploration of this phenomenon may provide insight into how mammalian cells regulate the expression of genetic material.

Proposed Course of Project:

Continuation, to achieve stated objectives.

Publications:

1) Lowy, D.R.: Glucocorticoid induced transformation of a mink cell line which contains a murine sarcoma viral genome (submitted).

2) Teich, N.M., Weiss, R.M., Green, G., and Lowy, D.R.: Susceptibility of murine teratoma cells to murine leukemic virus (submitted).

* U.S. GOVERNMENT PRINTING OFFICE : 1977 O-720-079/7361

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